Isotachophoresis of Bacterial Cells

by

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B.Biotech (Hons)

January 2017

Submitted in total fulfilment of the requirements for the degree of

Doctor of Philosophy

School of Physical Sciences-Chemistry

University of Tasmania

Declaration

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Sui Ching Phung

January 2017

Acknowledgements

It would not be possible to go through my PhD journey without the support and assistance from many individuals.

My foremost sincere thank you is to Professor Michael Breadmore, for being the first person that provided me a research opportunity in my undergraduate course that gradually led me to my honours and PhD under his supervision. Since 2011, Prof. Breadmore's supervision has provided me with great knowledge, ideas, encouragement, help and scientific understanding toward scientific works as well as a sense of confidence. All of these contributions have instilled me with opportunities to improve my research and communication skills. My PhD candidature was never a smooth journey, especially as a slow learner, I've encountered many obstacles such as instruments malfunctions, misunderstanding of the principle, yet, Prof. Breadmore has always been patient and tried his best to assist me. I apologised for that. I would also like to thanks Dr. Shane Powell, who has also been assisting me a lot in matter regarding microbiology and molecular biology since my honours (2012) and my PhD. Moreover, Dr. Powell has also been very patient and kind in helping me on my paper writing and grammar. Dr. Rosanne Guijt, who also helped me a lot in my PhD candidature in both science and non-science. Dr. Guijt has also taught me different ways to deal with failure throughout the duration of my PhD candidature. During the final stage of my PhD candidature, I've encountered some problems and Prof. Breadmore, Dr. Powell and Dr. Guijt have provided me with tremendous support to go through especially Dr. Guijt and Dr. Powell, for being my part time counsellor and listeners. I am truly grateful for the supports from them. Also, I would like to thank to Prof. Mirek for the support throughout the project.

I would also like to take this opportunity to thank Dr. Nai (Ryan) who has been a great mentor to me since undergrad, honours and my first year of my PhD. Dr. Nai was always been a strict mentor who gave me advice and instrument training on Beckman Coulter CE and assisted me with research skills and writing papers and presentations since undergrad.

Also, a great thank you to my colleagues who came into my working life, becoming my closest friends/family in UTAS/visiting fellows as a consequence of living abroad. They are Mei Yi Leow, Marina Lanz, Pavisara Nanthasurasak, Rob Haselberg, Curtis Ho, Reyne Pullen, Krystel Woolley, Alain Wuthrich, Sinead Currivan, Laura Tedone, Elisenda Vernet, Joan Marc Cabot, Niall MacDonald, Soohyun Park, Aliaa Shallan, Ying Chiing Yap and Petr Smejkal. I would like to thanks the staff in ACROSS and Chemistry for all the assistances and friendships since 2011. I would also like to take the opportunity to thank UTAS for the Scholarship support for the 3.5 years of my PhD candidature. Apart from my colleagues, a special thanks to Dr. Michelle Yap and Dr. Yuen Zhu for being a best friends who supports me and gives me a lot of positive advices whenever I faced issues and also taking some of their time to proof read my PhD thesis.

Finally, I would like to thank my family especially my father and mother for the support and encouragements. Without them, I would not have taken a huge step out of my comfort zone by moving to Tasmania alone. Without the encouragement from them, I would not be where I am. The family supports from them have helped me since I moved to Tasmania in 2010 for my academic studies. I appreciate the sacrifices they made that drive me to the completion of this PhD candidature.

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The following people and institutions contributed to the publication of work undertaken as part of this thesis:

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List of Publications and Presentations

1. Counter-pressure assisted ITP with electrokinetic injection under field amplified conditions for bacterial analysis (Chapter 2)

   S.C. Phung, Y.H. Nai, S.M. Powell, M. Macka, M.C. Breadmore

   Analytical and bioanalytical chemistry (2015) vol.407, p: 9665-7002.

   Doi: 10.1007/s00216-015-8838-4

2. Isotachophoretic fluorescence *in situ* hybridisation of intact bacterial cells. (Chapter 3)

   S.C. Phung, J.M. Cabot, S.M. Powell, R.M. Guijt , M. Macka, M.C. Breadmore

   Analytical Chemistry (2017). (Accepted on the 22nd May 2017).

   Doi: 10.1021/acs.analchem.7b00598

3. Microfluidic isotacphoretic fluorescence in situ hybridisation of bacterial cells (Chapter 4)

   S.C. Phung, Y.H. Nai, S.M. Powell, R.M. Guijt, M. Macka, M.C. Breadmore

   19th International Conference on Miniaturised Systems for Chemistry and Life Sciences (2015) p.1960-1962

4. Multimaterial 3D printing: Integrated electrodes for ITP of bacterial cells (Chapter 4)

   S.C. Phung, L. Feng, S.M. Powell, R.M. Guijt, M. Macka, M.C. Breadmore

   20th International Conference on Miniaturised Systems for Chemistry and Life Sciences (2016) p: 1081-1082.

5. Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips (2012-2014) (Chapter 1)

   M.C. Breadmore, R.M. Tubaon, A.I. Shallan, S.C. Phung, A.S. Abdul Keyon, D. Gstoettenmayr, P. Prapatpong, A.A. Alhusban, L. Ranjbar, H.H. See, M. Dawod, J.P. Quirino

   Electrophoresis (2015) vol:36. pp:36-61.

   Doi: 10.1002/elps.201400420

6. Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips (2014-2016) (Chapter 1)

   M.C. Breadmore, A. Wuethrich, L. Feng, S.C. Phung, U. Kalsoom, J.M. Cabot, M. Tehranirokh, A.I. Shallan, A.S. Abdul Keyon, H.H. See, M. Dawod, J.P. Quirino

   Electrophoresis (2016) Accepted 10th August 2016

   Doi: 10.1002/elps.201600331

7. Capillary Electrophoresis of Nucleic Acids: Nucleic Acids structure and mapping (chapter 1)

   S.C. Phung, D. Gstottenmayr, M.C. Breadmore, Y.H. Nai

   Encyclopedia of Analytical Chemistry (2015)

   Doi 10.1002/9780470027318.a1404.pub2

8. *Isotachophoresis (ITP) of Cells*

   S.C. Phung, Y.H. Nai, S.M. Powell, R.M. Guijt, M. Macka, M.C. Breadmore, **Poster presentation,** 40th International Symposium on High Performance Liquid Phase Separations and Related Techniques - HPLC (2013). 18th-21st November, (Hobart, Australia)

9. *Microfluidic fluorescence in situ hybridisation of cells using Isotachophoresis (ITP)*

   S.C. Phung, Y.H. Nai, S.M. Powell, R.M. Guijt, M.C. Breadmore, **Oral presentation**, 6th Australia and New Zealand Nano Microfluidic Symposium - ANZNMF (2015). 31st March - 2nd April (RMIT, Melbourne, Australia).

10. *Microfluidic isotacphoretic fluorescence in situ hybridisation of bacterial cells*

    S.C. Phung, Y.H. Nai, S.M. Powell, R.M. Guijt, M. Macka, M.C. Breadmore, **Poster presentation**, 19th International Conference on Miniaturised Systems for Chemistry and Life Sciences – μTAS (2015). 25th to 29th October, (Gyeongju, South Korea).

11. *Fluorescence in situ hybridisation of bacterial cells using Isotachophoresis (ITP)*

    S.C. Phung, Y.H. Nai, S.M. Powell, R.M. Guijt, M. Macka, M.C. Breadmore, **Poster presentation,** 7th Australia and New Zealand Nano-Microfluidics Symposium - ANZNMF (2016). 21st to 23rd March, (Griffith University, Brisbane, Australia).

12. *Multimaterial 3D printing: Integrated electrodes for ITP of bacterial cells*

    S.C. Phung, L. Feng, S.M. Powell, R.M. Guijt, M. Macka, M.C. Breadmore, **Poster presentation,** 20th International Conference on Miniaturised Systems for Chemistry and Life Sciences - μTAS (2016). 9th to 13th October (Dublin, Ireland).

13. *Multimaterial 3D printing: Integrated electrodes for ITP of bacterial cells*

    S.C. Phung, L. Feng, S.M. Powell, R.M. Guijt, M. Macka, M.C. Breadmore, **Oral presentation,** 2nd ACROSS Symposium on Advances in Separation Sciences - ASASS (2016). 29th November to 2nd December (Hobart, Australia).

List of Abbreviations

ABS	Acrylonitrile Butadiene Styrene
ASPCR	Allele Specific PCR
bp	base pair
BPE	Bipolar electrode
BWA	Biological warfare agent
CAD	Computer Aided Design
CAE	Capillary Array Electrophoresis
CCD	Charge-coupled Device
CE	Capillary Electrophoresis
CF	Counter flow
CFU	Colony Forming Unit
CGE	Capillary Gel Electrophoresis
cITP	Capillary Isotachophoresis
CMT1A	Charcot Marie Tooth 1A
CSE	Capillary Sieving Electrophoresis
Cy5	Cyanine 5
CZE	Capillary Zone Electrophoresis
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DzITP	Depletion zone ITP
dsDNA	Double stranded DNA
E. coli	*Escherichia Coli*
EDTA	Ethylenediaminetetra Acetic Acid
EKI	Electrokinetic Injection
ELISA	Enzyme Linked Immunosorbent Assay
EOF	Electroosmotic Flow

ESI	Electron spray ionisation
ET	Energy transfer
FAM	6-Carbocyfluorescein
FASI	Field Amplified Sample Injection
FDM	Fused Deposition Modelling
FISH	Fluorescence *In Situ* hybridisation
HDI	Hydrodynamic Injection
HEC	Hydroxyethyl cellulose
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
HHC	Heriditary Hemochromatosis
HPCE	High Performance Capillary Electrophoresis
HPLC	High Performance Liquid Chromatography
HV	High Voltage
i.d.	Internal Diameter
ISO	International Organisation of Standardisation
ITP	Isotachophoresis
LB	Luria Bertani
LE	Leading Electrolyte
LED	Light Emitting diode
LIF	Laser-induced Fluorescence
LOD	Limit of Detection
LOQ	Limit of Quantitation
LPA	Linear polyacrylmide
LVSS	Large Volume Sample Stacking
MB	Molecular Beacon
MCB	Moving Chelation Boundary
ME	Microchip Electrophoresis
MEKC	Micellar Electrokinetic Chromatography

MRB	Moving Reaction Boundary
MSB	Moving Substitution Boundary
MS	Mass Spectrometer
μCAE	Micro Capillary Array Electrophoresis
μTAS	Micro Total Analysis System
NA	Nucleic Acid
PAEKI	Pressure-Asssisted Electrokinetic Injection
P. aeruginosa	*Pseudomonas aeruginosa*
PBS	Phosphate Buffer Saline
PCB	Printed Circuit Board
PCR	Polymerase Chain Reaction
PDMA	Poly-N-N-dimethylacrylamide
PDMS	Polydimethylsiloxane
PEO	poly(ethylene oxide)
pITP	pseudo Isotachophoresis
PMT	Photomultiplier Tube
PMMA	Poly Methyl Methacrylate
PLA	Polylactic Acid
PVP	Polyvinylpyrrolidone
qPCR	real-time PCR
QqQ	Triple Quadupole
R6G	Rhodamine 6 G
RSD	Relative Standard Deviation
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic acid
S	Sample
SE	Spacer Electrolyte
SEF	Signal Enhancement Factor

SLA	Stereolithography
SNP	Single Nucleotide Polymorphism
SPE	Solid Phase Extraction
ssDNA	Single stranded DNA
STL	Standard Triangulation Language
STR	Short Tandem Repeats
SW	Sample Waste
TE	Terminating Electrolyte
tITP	transient Isotachophoresis
TRIS	Tris (hydroxymethyl)-aminomethane
UV	Ultraviolet
ZE	Zone Electrophoresis

Abstract

Currently, the standard method for bacterial enumeration is still the plate count method. The issues with this method includes it is time consuming, laborious and it is only able to detect culturable bacteria. While there have been alternative methods reported for bacteria detection, most of the alternative methods either suffer from poor sensitivity or selectivity. This thesis documents research on developing an alternative method for microbial detection using isotachophoresis (ITP) and to transfer this method to a 3D printed microchip with integrated electrodes for the possibility of fabrication of these integrated devices.

In the first part, a counter-pressure was applied to extend the duration of the field amplified sample injection (FASI) in ITP to improve the detection of bacterial cells. By using a universal nucleic acid dye, the DNA and RNA in *Escherichia coli (E. coli)* was stained, and focused into the narrow ITP boundary. The use of the counter-pressure allows more cells to be injected and focused into the stationary ITP boundary. The counter-pressure was then removed to allow the ITP band to move past the detector. To obtain the optimum conditions, negatively charged encapsulated fluorescent beads with a diameter of 0.51 μm were used as a model. The injection voltages, applied counter-pressure and the injection time was optimised and then transferred to *E. coli*. Using – 12 kV with a 1.3 psi counter pressure for 4 mins, followed by mobilisation and continued injection at – 6 kV, the limit of detection (LOD) was 78 cells/mL, resulting in an improvement of 4 when compared with FASI-ITP without counter-pressure.

The second part of the thesis focussed on an ITP method for rapid hybridisation of the 16S rRNA inside the bacterial cells with a fluorescence *in situ* hybridisation (FISH) probe. This approach uses a two-stage separation within a single ITP analysis. With the introduction of spacer ions and sieving matrix, the in-line FISH between the probe and *E. coli* occurs within the first 10% of the total capillary length during ITP. When the ITP band reaches the sieving

matrix in second section of the capillary, the spacer ions overtake the stained *E. coli* forming two discrete ITP bands. The first ITP band is the free FISH probe focussed between the leader and spacer ions and the second ITP band is the stained *E. coli* focussed between the spacer ions and terminator ions. This method was then studied for selectivity with the use of PseaerA probe on *Pseudomonas aeruginosa (P. aeruginosa)* and *E. coli* cells. The analysis results successfully showed that the PseaerA probe does not bind with *E. coli* using the ITP method. The next part was to study the efficiency of the hybridisation using the ITP method and compared with the off-line FISH labelled cells. Fluorescent microscopy counting and ITP results showed that the in-line FISH ITP method stains 50% of the total cells injected with a 2 mins counter-pressure injection in comparison to the off-line FISH ITP method. When 6.0×10^4 cells were injected (3 times higher than the LOD reported by Lantz *et al.* in 2008) for in-line FISH ITP, approximately 3.0×10^4 cells were stained. This result was confirmed by analysis of off-line staining of 3.0×10^4 cells/mL.

The final body of the thesis is the transfer of the ITP method into microchip for portability. This chapter is divided into two sections. In the first section, a cross PDMS chip with 50 μm width and 42 μm depth was used for in-line ITP of intact bacterial cells. 7% PVP was selected as sieving matrix instead of 1.8% HEC due to the high viscosity of the polymer to be mechanically injected into the channel of the PDMS chip. With the optimisation of DMSO concentration to allow the probe to enter the cells for hybridisation during ITP in the PDMS chip, the total analysis time was reduced from 30 min (capillary) to 4 min (PDMS chip). Second part of this chapter was to study the use of a 3D printer to print a multimaterial chip with integrated electrodes for ITP of bacterial cells. A FDM printer (Felix 3.0 dual extruder head) was used to print a microfluidic chip using transparent acrylonitrile butadiene styrene (ABS) base material for the straight channel. The second extruder was used to deposit the conducting PLA electrodes embedded into the devices. The electrical resistance of the 3D integrated electrodes was examined and compared with in-house Pt electrode using the same chip geometry. While joule heating was observed when 600 V was applied onto the

3D printed chip, the 3D printed chip were able to carry a stable current up to 380 μA that is suitable for ITP. An ITP band of *E. coli* stained off-line with SYTO 9 was observed in the 3D printed chip. The rapid quantification of the device was examined and the detection limits of the cells using the PMT devices was 4.0 x 10^4 cell/mL which is higher than previously reported in capillaries most likely due to the LED fluorescence light used and the small sample volume used in the chip reservoir. While optimisation of the 3D printed chip is required, the 3D printer allows for the print of microchip with integrated electrodes using commercially available thermoplastic materials that can be used for mass productions of the chip when compared to PDMS chip. Moreover, the total time for printing a chip is 2 hours with the cost per chip is AUD $0.50.

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Chapter 1 has been removed for copyright or proprietary reasons.

Some segments of the chapter have been published in two review papers and a book chapter, (see p. vii for details).

2. Counter-pressure assisted ITP with electrokinetic injection under field amplified conditions for bacterial analysis

Declarations: This chapter has been published in Analytical Bioanalytical Chemistry journal as described in the statement of Co-Authorships and lists of publications and presentation. The material in this chapter is replicated from the publication with only alteration in the format for the thesis.

2.1 Abstract

Counter-pressure was used to extend the duration of field-amplified sample injection in isotachophoresis (FASI-ITP) in order to improve the detection of bacterial cells. Using 0.51-μm negatively charged encapsulated fluorescent beads as a model, the counter-pressure, injection and separation voltages, and times were optimised. Using 6 min, 1.3 psi counter-pressure FASI-ITP injections at - 12 kV followed by mobilisation of the ITP band with continued injection at - 6 kV, the limit of detection (LOD) for *Escherichia coli* was improved to 78 cells/mL, a factor of 4 when compared with FASI-ITP without counter-pressure.

2.2 Introduction

Outbreaks of pathogenic bacteria can be anything from inconvenient to fatal and are often costly to society. Therefore, rapid and specific detection of potentially hazardous microorganisms has become important in many sectors including (drinking) water [1], food [2], pharmaceutical industries [3], agriculture, aquaculture [4-6] and pathology [3]. The plate count assay uses either general or selective media and is still the most common and standard method for enumeration of bacterial cells because it is inexpensive and its sensitivity is as low as 1 colony forming unit (CFU) per mL [2]. Unfortunately, it requires a long assay time

(48 hr incubation) [7], specialised personnel [8] and is only able to detect viable and culturable bacteria [2]. Despite many alternative methods that can be used for bacterial detection, there is currently no method capable of replacing the plate count method in terms of its cost and sensitivity [2, 9].

Capillary electrophoresis (CE) gained significant interest as an alternative method for separation and analysis of microorganisms in the late 90s [8, 10-12]. Hjerten was the first to report the electromigration of viruses through a capillary when an electric field was applied for virus purification and used the same set-up for *Lactobacillus casei* [13]. Ebersole and McCormick were the first to demonstrate the separation of bacteria using capillary zone electrophoresis (CZE) [14]. Although the separation of bacteria has attracted considerable interest, Petr *et al.* stated that the sensitivity of CE is a major limitation [11] because the required sensitivity is as low as 1000 CFU/100 mL [15] for irrigation water and 1 CFU/ 100 mL drinking water [16]. To place this into context, assuming that a single cell is detected from a typical hydrodynamic injection of 10 nL, this gives a detection limit of 100 000 cells/mL, and it is the small volume that is injected that is the main problem.

To increase the volume injected into the capillary, Lantz *et al.* used a blocking plug to focus all of the injected cells into a narrow zone thus allowing eight times more sample to be injected [8]. They showed the detection of a single cell in 158 nL giving a limit of detection (LOD) of 6329 cells/mL [8]. Petr *et al.* used a large i.d. capillary (100, 250 and 320 μm) with large volume sample stacking (LVSS) to increase the injection volume and 120-fold enhancement was obtained using 320 μm i.d. capillary [17]. However, the major limitation of a hydrodynamic injection (HDI) is that the volume injected can never be greater than the total capillary volume [12].

Electrokinetic injection (EKI) can be used to overcome the limitation of HDI because the analytes are injected through the combination of their electrophoretic mobility and the electroosmotic flow (EOF) [18]. Using methods such as field amplified sample injection (FASI), 1000 to 100 000–fold enhancements have been obtained alone or in

combination with transient isotachophoresis (tITP) [19-21]. Oukacine *et al.* reported the use of ITP in combination with HDI and EKI for quantitation of bacteria [7]. They were able to focus and mobilise the bacteria into a relatively sharp peak with a 500-fold enhancement giving a LOD of 20 000 cells/mL [7, 10]. Previously, we used ITP under FASI conditions to concentrate *Escherichia coli (E. coli)* after staining with SYTO 9 and obtained a LOD of 135 cells/mL [12]. While more sensitive than the other CE methods, it is still limited by the small 100 μL volume of sample from which the cells are injected.

To address these issues, a counter-pressure could be used to allow longer injections and a greater number of cells to be injected from the sample vial. ITP with a counter-pressure was reported by Reinhoud *et al.* in 1993 [22] as a way to increase the volume injected without compromising the capillary length available for separation. Whilst the authors demonstrated the counter-pressure method for the analysis of anions and cations [22, 23] the hydrodynamic injection continued to limit the volume of sample injected. Feng *et al.* used pressure-assisted electrokinetic injection (PAEKI) with CZE/MS for analysis of DNA nucleotides [24]. The nucleotides were electrokinetically injected and a pressure was used to counter the EOF migrating towards the inlet, thus allowing more analytes to be injected and focused in the capillary before separation. An enhancement of 5000-fold was achieved compared to normal CZE/MS. Dawod *et al.* reported the use of counter-pressure during electrokinetic supercharging (EKS) for on-line preconcentration of seven non steroidal anti-inflammatory drugs in water samples and managed to obtained a 11 800-fold enhancement factor giving the LODs of 10.7 – 47.0 ng/L [25]. Breadmore used the EOF to balance the velocity of an ITP boundary and reported a LOD 10 000 times lower than a hydrodynamic injection [26] later extended to a low conductivity sample using a suitable terminating electrolyte to give a 100 000-fold reduction [20] and then high conductivity sample containing a suitable leader [27].

In this study, we performed ITP under FASI conditions with a counter-pressure to counter the movement of the ITP boundary and allow more cells to be electrokinetically injected from the sample into the capillary. After a set time, the pressure was removed

allowing the ITP band to pass through a fluorescence detector for quantitation. The method was optimised using fluorescent beads and transferred to bacterial cells, with sensitivity improved by a factor of 4 when compared to our previously published ITP method [12]. The counter-pressure approach here gave a LOD of 78 cells/mL, the lowest concentration LOD for bacterial cells analysis reported by any electrophoretic method.

2.2 Materials and Methods

2.2.1 Chemicals

Tris(hydroxymethyl)aminomethane) (Tris), ≥ 99.95%, polyvinylpyrrolidone (PVP) (M_w 1300 kDA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 99.5%, 1 M Tris HCl (pH 8.0) were purchased from Sigma Aldrich (St. Louis, USA). Fluorescent carboxyl-functionalised microspheres (mean diameter: 0.51 μm) with internal fluorophore (excitation 480 nm, emission 520 nm) were purchased from Bangs Laboratories Inc. (Indiapolis, USA). Luria Bertani media (LB, 1 L LB – 10 g tryptone, 5 g yeast extract, 10 g NaCl) and LB agar plates were prepared in house.

Tryptone, yeast extract and Phosphate Buffered Saline (PBS) were purchased from Oxoid (Hampshire, England). Sodium chloride was purchased from Univar (Sevenhill, Australia). Agar was purchased from Gelita (Beaudesert, Australia). SYTO 9 was purchased from Invitrogen (Mulgrave, Australia).

2.2.2 Electrolytes

For all ITP experiments, the leading (LE) electrolyte was 50 mM Tris HCl (pH 8.0) with 0.5 % w/v of PVP and the terminating (TE) electrolyte was 0.5 – 5 mM Tris HEPES (pH 7.8) with a 1:1 ratio. Both LE and TE solutions were prepared in 18 MΩ Milli-Q water (Millipore, Bedford, MA, USA). The pipettes used were sprayed with Rnase to remove contaminations. Pipette tips used were purchased from Molecular Bioproduct with aerosol resistant tips where the tips are pre-sterilised before packaging. The microtubes and electrolyte solutions used were autoclaved prior using. For plating, aseptic technique was used.

2.2.3 Fluorescent beads, bacteria growth and sample preparation

Negatively charged encapsulated fluorescent beads with a mean size of 0.51 μm were used as a model analyte as their size is similar to that of *E. coli* [12, 15]. A 100x diluted beads stock solution was prepared with 10 μL of the supplied fluorescent beads stock added to 990

μL of 5 mM Tris HEPES. All solutions were wrapped with aluminium foil and kept in the fridge at 4 ° C until required.

The model strain *E. coli* TOP10 (Invitrogen, Mulgrave, Australia) was cultured in solid LB media at 37°C and, when necessary, in liquid LB broth at 37°C with shaking. For ITP experiments, an *E. coli* colony was inoculated into sterile LB broth and incubated overnight. To harvest the cells, 10 mL of the overnight cell culture was transferred to a sterile 15 mL centrifuge tube and spun at 3600 rpm at 5 min at room temperature (model: Universal 16 A, Hettich, Zentrifugen, Tuttlingen, Germany). The supernatant was carefully discarded and cell pellet was resuspended in sterile PBS. This suspension was stored at 4 °C until required and remained viable for up to one week. Cell number of *E. coli* in colony forming unit (CFU) (CFU unit approximately to cells), was determined by the plate count method on LB agar

2.2.4 Sample preparation

Fluorescent beads: The fluorescent beads stock at 100x dilution was further diluted down to 20 000x dilution in 5 mM TE and the final volume of each dilution was 2000 μL. The beads number of a 20 000x dilution was determined to be 6.575 x 10^6 particles/mL using a Petroff-Hauser Chamber according to manufacturer's manual instructions..

Bacterial cells: 100 μL of *E. coli* in PBS was centrifuged at 3600 rpm for 5 min at room temperature (Model: Eppendorf 5424, Hamburg, Germany). The supernatant was discarded, cell pellet was resuspended in 100 μL of 5 mM TE and mixed by gentle vortex for 20 s. This washing step was repeated once. Serial dilutions were performed on this cell suspension using 5 mM TE with a final volume of each dilution of 1000 μL.

Cells were stained by adding 100 μL *E. coli* cells in TE to 850 μL sterile TE in a sterile microtube, then 50 μL of 20 μM SYTO 9 was added to give a final concentration of 1 μM SYTO 9.

The microtubes were wrapped with aluminium foil and allowed to incubate at room temperature for at least 30 min prior to analysis. 100 µL SYTO 9 stained *E. coli* cell sample was vortexed before aliquot into a sterile PCR vial used for each analysis and was replaced after each injection.

2.2.5 CE Instrumentation

ITP studies were performed using a Beckman Coulter P/ACE MPQ Capillary Electrophoresis System equipped with 488-nm Argon laser module. Experiments were conducted at 25°C using an unmodified fused silica capillary of 50 µm i.d. (Polymicro Technologies, AZ, USA) with a total length of 40 cm (effective length to detector, 30 cm). A different sample vial and cap with spring that holds the PCR vials was used for sample injection allowing the capillary and electrodes to reach the end of the tube with equal distribution of cells to be injected into the capillary Data was collected and analysed using the 32 Karat software version 8.

2.2.6 Capillary conditioning and isotachophoresis

Prior to analysis, a new capillary was preconditioned at 275,790 Pa (40 psi) in the following procedure: 1M NaOH (15 min) , Milli-Q water (4 min), 1M HCl (10 min), Milli-Q water (3min), 1% w/v of PVP in 50 mM Tris- HCl, pH 8.0 (20 min) and Milli-Q water (3min). Each analysis began by flushing with LE for 6 min prior to placing the vials with beads/stained cells suspended in TE at the inlet and applying the ITP voltage (-12kV, 300V/cm with counter-pressure (psi), follow by separation at 6kV, 150V/cm).

2.3 Results and Discussions

2.3.1 Isotachophoresis (ITP) system

The capillary length is one of the HDI limitations that restrict the volume of sample that can be injected into the capillary. While this can be overcome by EKI, the movement of the ITP boundary through the capillary during injection limits the injection time, and/or the capillary length available for subsequent separation in transient systems [25]. A counter-pressure can be used to allow for longer EKI, and hence the injection of more sample, by holding the ITP band stationary leaving sufficient capillary length for separation [20]. Figure 2.1 shows the counter-pressure ITP system. At the start (figure 2.1 (i)), the capillary is filled with LE. The inlet sample vial contains the cells suspended in the TE. When the voltage is applied (ii), the TE ions and sample are electrokinetically injected into the capillary. The ITP system is created by the LE and TE ions, with the cells stacking at the boundary between LE and TE. As soon as the voltage is applied, a counter-pressure is also applied to hold the TE and the cell boundary at a fixed position within the capillary. Because cells have higher mobility than the TE, they are continuously injected and focussed at the cell band at the LE/TE interface. By holding the boundary stationary, more cells can be injected and focussed within the sample band as shown in the third image (figure 2.1 (iii)). After a sufficient period of time, the counter-pressure is removed to allow the ITP to move toward the outlet and the cell band to pass through the detector. In this work, the optimal voltages for EKI and mobilisation of ITP band toward the detector were first studied prior to optimising the counter-pressure.

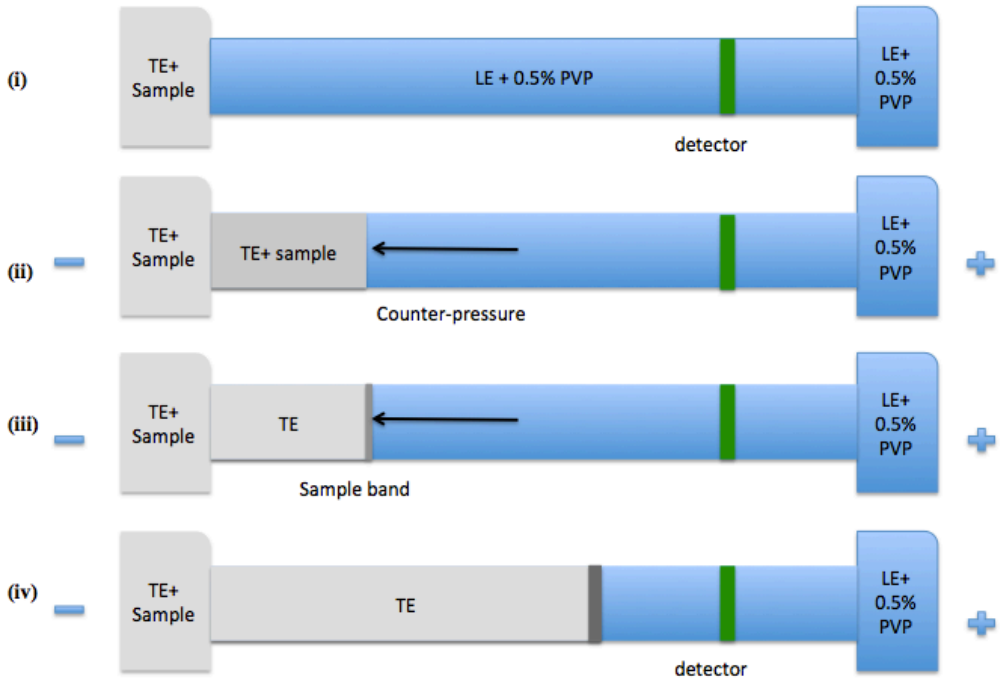

Figure 2.1: Schematic of counter pressure (ITP). (i) Capillary is filled with leading electrolyte (LE) while sample is suspended in the terminator electrolyte (TE) in inlet sample vial. (ii) Voltage of (-12kV) applied together with a counter-pressure (1.3psi) allowing samples in TE to be electrokinetically injected into the capillary. (iii) The sample cells are separated from the TE forming a narrow sharp ITP band between the LE and TE interface at a fixed position due to the presence of counter-pressure. (iv) Counter-pressure was removed, and the voltage was reduced to -6kV to allow the ITP band to move along the capillary to the detector.

2.3.2 Voltages for injection and mobilisation of the ITP band

With the fluorescent particles suspended in TE, varying the injection voltage will influence the electric field distribution over the sample as well as the velocity of the ITP boundary. As the injection is performed under field amplified conditions, this movement will reduce the electric field strength over the sample. Using a single point detector, it is not possible to correlate any change in peak height/area to either of these variables. Feng *et al*. reported the use of various voltages from -8 to -13 kV under a fixed external pressure of 50 mbar with PAEKI [24] with -13kV being the optimum. Dawod *et al.* studied -10 to -20 kV with 50 mbar counter-pressure and found that -16 kV the optimum [25]. In our previous study, we reported the use of -16kV to continuously inject the sample into the capillary but noticed that at this high a voltage the PVP coating was unstable [12]. Thus, -12 kV was selected as the injection voltage to inject the sample into the capillary. To optimise the ITP separation voltage, a fixed (not optimised) hydrodynamic counter-pressure of 8274 Pa (1.2 psi) was applied with a -12 kV injection voltage for 4 min. After the injection, the counter-pressure was removed, the capillary inlet placed in TE and the separation voltage varied. Figure 2.2 shows the average peak area of three replicates with different voltages used. From the figure 2.2, as the voltage is lowered, the peak area increases due to the band spending a longer time in the laser, with the peak area reaching a maximum at -6 kV. At -4 kV, the peak area decreased most likely due to photobleaching. Therefore, we conclude that -6 kV is the optimal voltage for the ITP band to pass through the detector with maximum peak area.

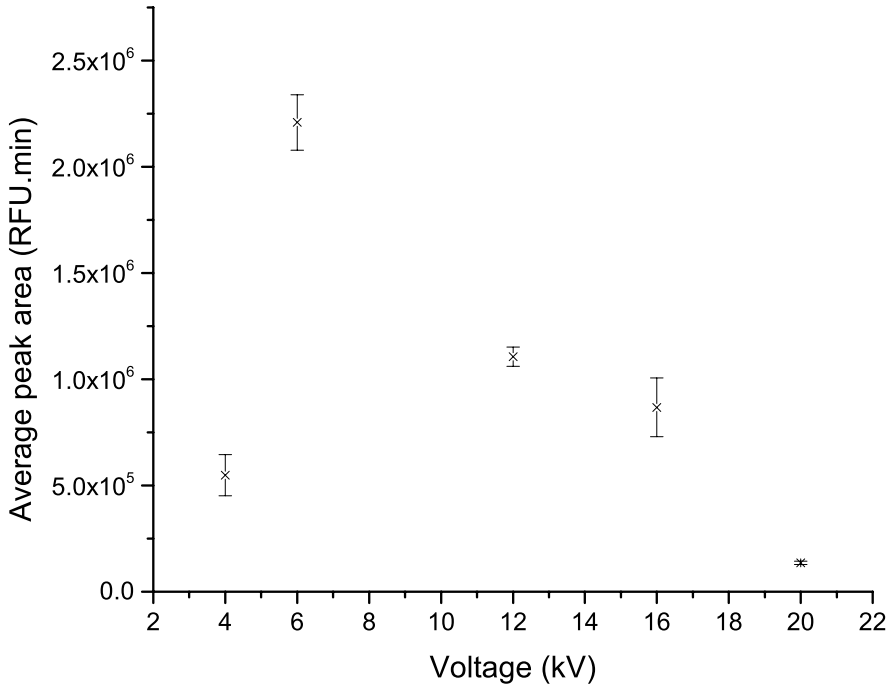

Figure 2.2: The effect of varying post-injection voltage on the detector response of fluorescent beads. Sample consisting of 6.58 x 10^6 particles/mL. Experimental conditions: 40 cm x 50 µm i.d. bare fused silica capillary (detection window at 30 cm). At each analysis, the capillary was filled with LE: 50 mM Tris HCl + 0.5% w/v PVP (pH 8.0), for 5 min followed by injections of beads in 100 µL, TE: 5 mM Tris HEPES (pH 7.8) at field strength of 300V/ cm (-12 kV) with 1.2 psi counter-pressure for 4 min, followed by 100 - 500 V/cm (- 4- 20 kV) at reversed polarity.

2.3.3 Effect of counter-pressure and time injection study

Using an injection voltage of -12 kV for the reasons described above, we studied the effect of different counter-pressures applied within 4 min of injection. Figure 2.3A shows the average peak area of three replicates when different counter-pressures were applied. The result shows that peak area correlates with increasing counter-pressure and reached a plateau after 8963 Pa (1.3 psi) and with no significant difference between 8963 – 11721 Pa (1.3 to 1.7 psi). However the peak area decreased at 14479 Pa (2.1 psi) because the counter-pressure resulted in a hydrodynamic flow greater than the electrophoretic velocity of the beads,

preventing the beads from being injected into the capillary. As such, a counter-pressure of 8963 Pa (1.3 psi) was selected as an optimum pressure for the remainder of this work.

Using this optimised pressure-voltage combination, it is possible to stop the movement of the ITP boundary. This can be seen from the current traces shown in Figure 2.3B where a current of -10.0 μA is reached after about 5 min after which the current does not change significantly indicating that the composition of the capillary is no longer changing and the ITP boundary has become stationary. Figure 2.3C shows the observed peak area of the particles for the same injection times where it can be seen that the peak area increased linearly as the injection time increased up to 20 min and did not plateau. Injection times longer than this were not examined to keep total analysis times less than 30 min.

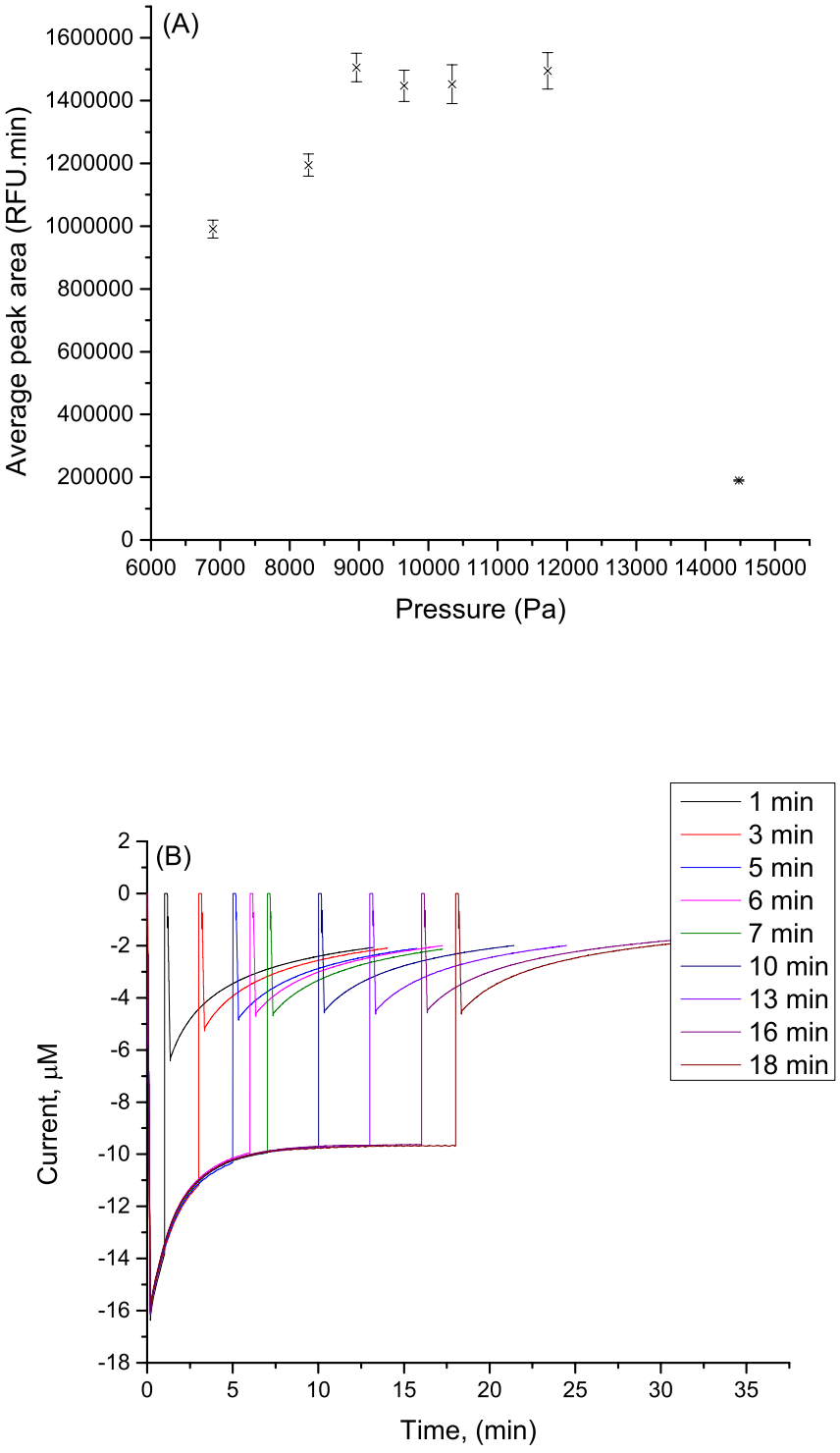

Figure 2.3: (A) Effect of varying counter-pressure with a fixed injection voltage of -12 kV. Sample consisting of 6.58 x 10^6 particles/mL (B) Current profiles of different injection times with fixed counter-pressure (8963 Pa) and injection voltage (-12 kV). Sample consisted of 6.58 x 10^6 particles/mL.

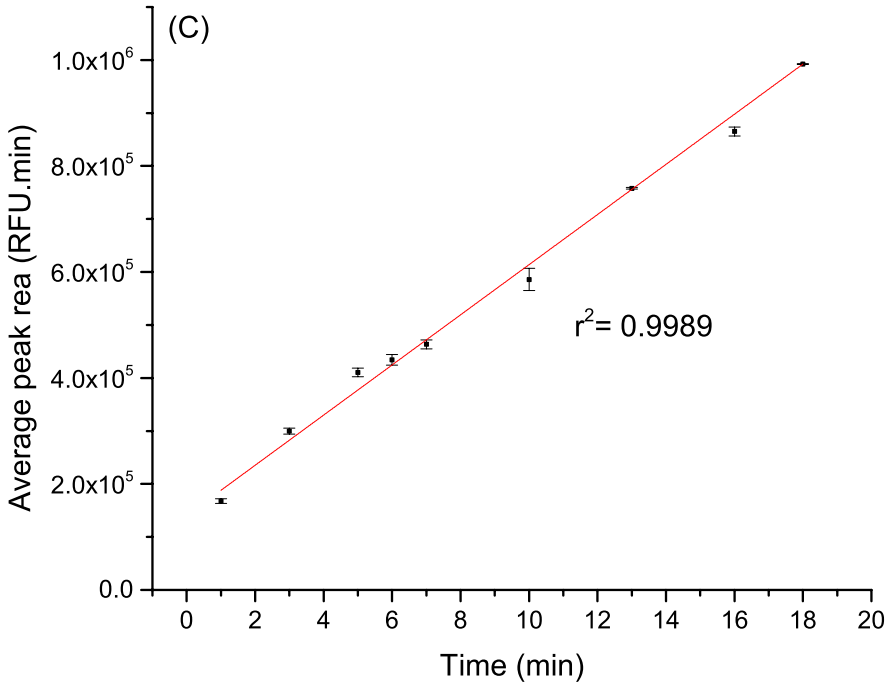

Figure 2.3: (C) Effect of different injection times under constant injection voltage (-12 kV) with fixed counter- pressure (1.3 psi). Experimental conditions: Experimental conditions: 40 cm x 50 µm i.d. bare fused silica capillary (detection window at 30 cm). At each analysis, the capillary was filled with LE : 50 mM Tris HCl + 0.5% w/v PVP (pH 8.0), for 5 min followed by injections of beads in 100 uL, TE: 5 mM Tris HEPES (pH 7.8) at field strength of 300V/ cm (-12 kV) with counter-pressure (6895-14479 Pa) for varying time (1-18 mins), followed by 150V/cm (-6 kV) at reversed polarity.

2.3.4 Continuous injection

The Santiago's group has demonstrated that a sample can be continuously injected from the TE, rather than changing to a separate TE vial [28-32]. When compared to a discrete fixed-time injection of the sample, this should help to provide lower detection limits. To determine whether this was the case, the peak areas for discrete and continuous injection of the fluorescent beads was determined. Discrete injections were conducted at -12 kV (8963 Pa counter-pressure), followed by replacement of the sample vial with a TE vial together, and a voltage of -6 kV applied. For continuous injection, a voltage of -12 kV was applied together with 8963 Pa counter-pressure on the sample vial for the injection time, After injection, the sample vial was not replaced and the voltage was reduced to -6 kV to allow the ITP band to move toward detector. In both discrete and continuous injection mode the injection times studied were 0, 1, 3, 7, 10 and 16 min with the results shown in figure 2.4. From the figure, there is a significant difference at low injection times, and logically it took about a 3 min discrete injection at -12 kV to reach the same detector response as the continuous injection at -6 kV (the peak takes approximately 6 min to reach the detector). Interestingly, there is a higher peak area response with the discrete injection at 10 and 16 min than with a continuous injection. This is counter intuitive and currently we do not have any explanation for this phenomenon, but believe it may be attributed to either settling of the particles over injection time or may be related to a loss of the field amplified region over the sample with the continuous approach.

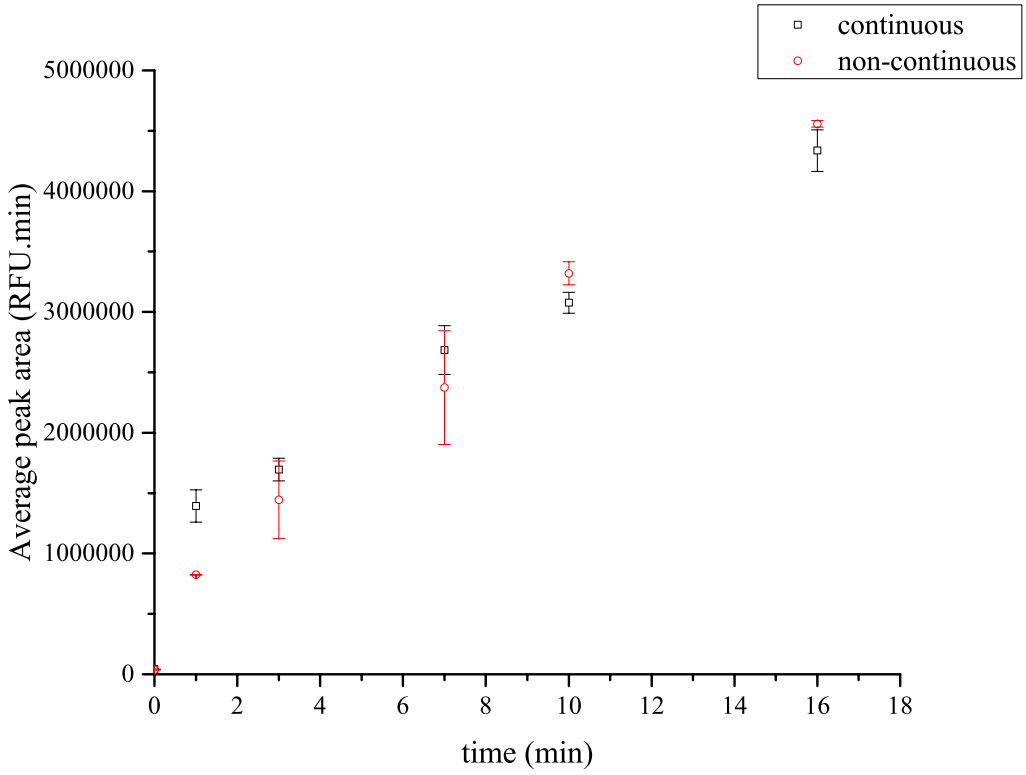

Figure 2.4: Study of continuous and non - continuous injection using optimised conditions. Sample consisting of 6.58 x 10^6 particles/mL. Experimental conditions: Experimental conditions: 40 cm x 50 µm i.d. bare fused silica capillary (detection window at 30 cm). At each analysis, the capillary was filled with LE : 50 mM Tris HCl + 0.5% w/v PVP (pH 8.0) for 5 min followed by injections of beads in 100 uL, TE: 5 mM Tris HEPES (pH 7.8) at field strength of 300V/ cm (-12 kV) with 1.3 psi counter-pressure for 6 min, followed by 150V/cm (-6 kV) at reversed polarity.

2.3.5 Counter-pressure ITP on *E. coli*

The optimised injection conditions - continuous injection using FASI-ITP at -12 kV with 8963 Pa (counter-pressure) for 7 min followed by the use of -6 kV – were applied on *E. coli* suspended in TE (5 mM Tris HEPES). However, the injection time was adjusted to 6 min as we discovered when using injections longer than 7 min, bubbles formed within sample vial that affected the repeatability. This was not observed using the beads.

Figure 2.5A shows two linear calibration data for varying *E. coli* concentrations ranging from 380 – 38 000 cells/mL stained with SYTO 9 for the counter-pressure ITP compared to the ITP approach we previously published [12]. The LOD calculated for ITP was 322 cells/mL, which is 2.3 times higher than our previous report. This discrepancy with previous work is due to a different CE-LIF instrument being used for this study [12]. Nevertheless, the LOD calculated for counter-pressure ITP mode was found to be 78 cells/mL, an improvement factor of 4. We believe the blank peak is a result of fluorescein contamination, which was used to calibrate the instrument, despite washing the electrodes and cleaning the instrument thoroughly. To account for this contamination, a blank peak was conducted prior to each analysis, and the average peak heights/areas were used. To verify that the LOD can be distinguished from blank, we prepared a cell suspension of 100 cells/mL (close to the calculated LOD) stained with 1 μM SYTO 9. Figure 2.5B shows the overlayed isotachopherograms of both blank and 100 cells/mL. The peak area and peak height of the blank and cells can be distinguished as the average peak area of the blank was 29960 ± 3505 (n=3) while for 100 cells/mL it was 41330 ± 3473 (n=3). Surprisingly, we did not observe a step response in detection signal when dealing with this low number of cells. The %RSD for 100 cells was calculated to be 7.3% while the %RSD for the blank was 8.8% (n=5). We speculate that this could be because we are detecting cells by staining the nucleic acids inside the cells (DNA+RNA) with the total nucleic acid content of the call varying slightly depending on the growth state of the cells. This introduces some variability and inaccuracy in the quantitation of low cell numbers, however at acceptable levels.

The sample volume used in the experiments above was only 100 µL, which at a cell concentration of 100 cells/mL means that we are detecting 10 cells or less. To determine the number of cells being injected into the capillary at these lower cell concentrations, cells were serially diluted to 60 cells/mL in 2 mL of 5 mM Tris HEPES without SYTO 9. Six 100 µL replicate suspensions were plated onto LB agar, labelled and incubated at 37 °C overnight. An additional six replicates were injected into the CE with the aforementioned optimised conditions. The solution remaining in the sample vial was transferred onto LB agar and incubated overnight at 37 °C. From the plate count results, at least 50% of the cells were injected and focussed in the ITP band, meaning that at the LOD, we were detecting approximately 5 of the cells. In addition from the plate count results, there was no influence of non-specific adsorption of bacterial cells onto the surface of the PCR tube/capillary as the cells were fully transferred onto the agar plate for counting. Further improvements in the efficiency of cell injection to more effectively inject all of the cells from a specified volume will see the detection limits lowered further.

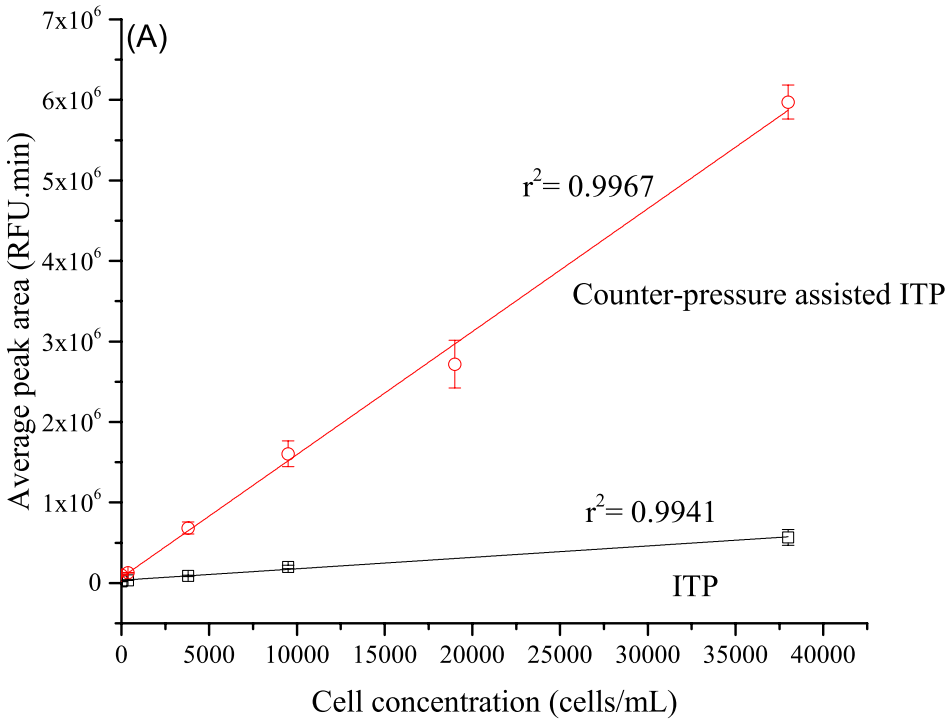

Figure 2.5: (A) Calibration lines of normal ITP vs optimised counter-pressure ITP of *E. coli* cells at concentrations of 380 - 38,000 cells/mL.

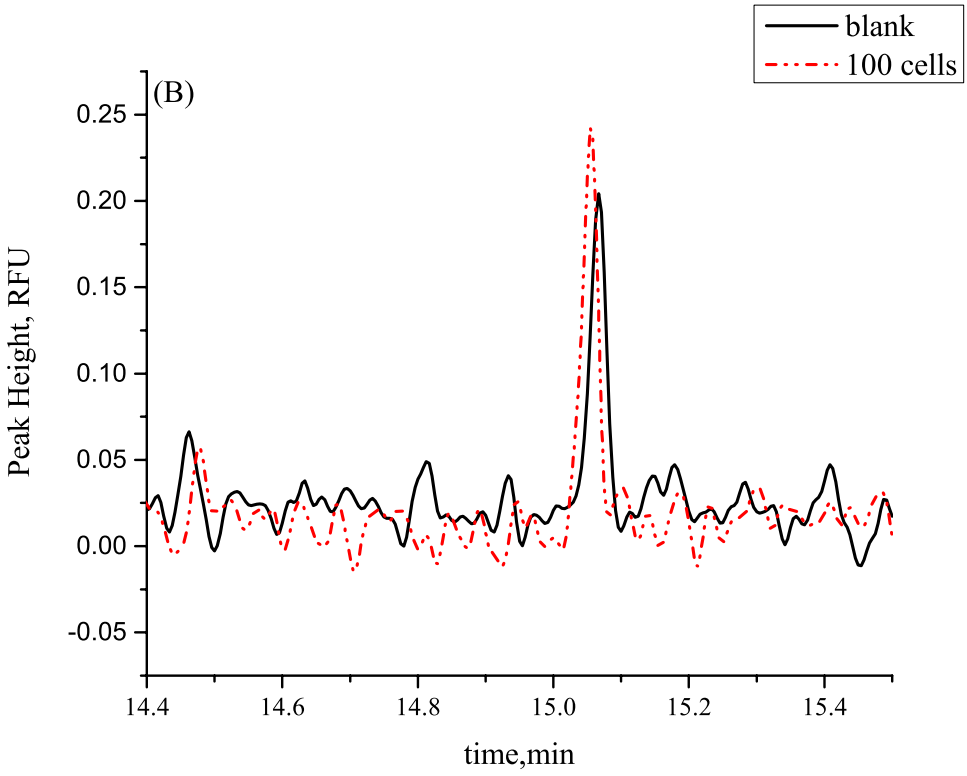

Figure 2.5: (B) Isotachopherogram of blank and 100 cells/mL. Experimental conditions: 40 cm x 50 µm i.d. bare fused silica capillary (detection window at 30 cm). At each analysis, the capillary is filled with LE: 50 mM Tris HCl + 0.5% w/v PVP (pH 8.0), for 5 min followed by injections of *E. coli* in 100 uL, TE: 5 mM Tris HEPES (pH 7.8) at field strength of 300 V/ cm (12 kV) with 1.3 psi counter-pressure for 6 min, followed by 150 V/cm (6 kV) at reversed polarity. Normal ITP mode: experimental conditions the same as describe by [12].

2.4 Concluding remarks

The application of capillary electrophoresis for rapid detection of cells is limited by a lack of ability to detect a few cells in a large volume. Here, the sensitivity was enhanced by the use of counter-pressure to immobilise the ITP zone during injection to allow longer injections and more efficient introduction of the cells into the capillary. The detection limits were 4 times lower when compared to an identical approach without the counter-pressure, with a detection limit of 78 cells/mL. Using a sample volume of 100 μL, we estimated that approximately 5 cells were injected and detected in the capillary. The analysis time per sample has increased from 5 min to 17 min, but new approaches for sample introduction may alleviate this issue.

2.5 References

1. Emmerson AM (2001) Emerging waterborne infections in health-care settings. Emerging infectious diseases 7:272–276.

2. Jasson V, Jacxsens L, Luning P, et al. (2010) Alternative microbial methods: An overview and selection criteria. Food microbiology 27:710–730.

3. Peters RPH, van Agtmael MA, Danner SA, et al. (2004) New developments in the diagnosis of bloodstream infections. The Lancet Infectious diseases 4:751–760.

4. Al-Harbi AH, Naim Uddin M (2004) Seasonal variation in the intestinal bacterial flora of hybrid tilapia (Oreochromis niloticus×Oreochromis aureus) cultured in earthen ponds in Saudi Arabia. Aquaculture 229:37–44.

5. Alves Resende J, L Silva VAN, Oliveira Fontes CAU, et al. (2012) Multidrug-Resistance and Toxic Metal Tolerance of Medically Important Bacteria Isolated from an Aquaculture System. Microbes and Environments 27:449–455.

6. Paillard C, Le Roux F, Borrego JJ (2004) Bacterial disease in marine bivalves, a review of recent studies: Trends and evolution. Aquatic Living Resources 17:477–498.

7. Oukacine F, Quirino JP, Garrelly L, et al. (2011) Simultaneous Electrokinetic and Hydrodynamic Injection for High Sensitivity Bacteria Analysis in Capillary Electrophoresis. Analytical chemistry 83:4949–4954.

8. Lantz AW, Bao Y, Armstrong DW (2007) Single-Cell Detection: Test of Microbial Contamination Using Capillary Electrophoresis. Analytical chemistry 79:1720–1724.

9. Rosmini MR, Signorini ML, Schneider R, Bonazza JC (2004) Evaluation of two alternative techniques for counting mesophilic aerobic bacteria in raw milk. Food Control 15:39–44.

10. Oukacine F, Garrelly L, Romestand B, et al. (2011) Focusing and mobilisation of bacteria in capillary electrophoresis. Analytical chemistry 83:1571–1578.

11. Petr J, Maier V (2012) Analysis of microorganisms by capillary electrophoresis. TrAC Trends in Analytical Chemistry 31:9–22.

12. Phung SC, Nai YH, Powell SM, et al. (2013) Rapid and sensitive microbial analysis by capillary isotachophoresis with continuous electrokinetic injection under field amplified conditions. Electrophoresis 34:1657–1662.

13. Hjerten S, Elenbring K, Kilár F, et al. (1987) Carrier-free zone electrophoresis, displacement electrophoresis and isoelectric focusing in a high-performance electrophoresis apparatus. Journal of chromatography A 403:47–61.

14. Ebersole RC, McCormick RM (1993) Separation and isolation of viable bacteria by capillary zone electrophoresis. Biotechnology (NY) 11:1278–1282.

15. Unkovich M, Stevens D, Ying G-G, Kelly J (2004) Impacts on crop quality from irrigation with water reclaimed from sewage. 1–62.

16. NHMRC, NRMMC (2011) *Australian Drinking Water Guidelines Paper 6 National Water Quality management Strategy*. National Resource management Ministerial Council, COmmonwealth of Australia, Canberra.

17. Petr J, Jiang C, Sevcik J, et al. (2009) Sterility testing by CE: A comparison of online preconcentration approaches in capillaries with greater internal diameters. Electrophoresis 30:3870–3876.

18. Breadmore MC (2009) Electrokinetic and hydrodynamic injection: making the right choice for capillary electrophoresis. Bioanalysis 1:889–894.

19. Breadmore MC (2012) Capillary and microchip electrophoresis: Challenging the common conceptions. Journal of chromatography A:1221:42–55.

20. Breadmore MC, Quirino JP (2008) 100 000-Fold Concentration of Anions in Capillary Zone Electrophoresis Using Electroosmotic Flow Controlled Counterflow Isotachophoretic Stacking under Field Amplified Conditions. Analytical chemistry 80:6373–6381.

21. Breadmore MC, Dawod M, Quirino JP (2010) Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips (2008-2010). Electrophoresis 32:127–148.

22. Reinhoud NJ, Tjaden UR, van der Greef J (1993) Automated isotachophoretic analyte focusing for capillary zone electrophoresis in a single capillary using hydrodynamic back-pressure programming. Journal of chromatography A 641:155–162.

23. Reinhoud NJ, Tjaden UR, van der Greef J (1993) Strategy for Setting Up Single-Capillary Isotachophoresis Zone Electrophoresis. Journal of chromatography A 653:303–312.

24. Feng Y-L, Zhu J (2006) On-Line Enhancement Technique for the Analysis of Nucleotides Using Capillary Zone Electrophoresis/Mass Spectrometry. Analytical chemistry 78:6608–6613.

25. Dawod M, Breadmore MC, Guijt RM, Haddad PR (2009) Counter-flow electrokinetic supercharging for the determination of non-steroidal anti-inflammatory drugs in water samples. Journal of chromatography A 1216:3380–3386.

26. Breadmore MC (2008) Unlimited-volume stacking of ions in capillary electrophoresis. Part 1: Stationary isotachophoretic stacking of anions. Electrophoresis 29:1082–1091.

27. Breadmore MC (2010) Journal of Chromatography A. Journal of chromatography A 1217:3900–3906.

28. Schoch RB, Ronaghi M, Santiago JG (2009) Rapid and selective extraction, isolation, preconcentration, and quantitation of small RNAs from cell lysate using on-chip isotachophoresis. Lab on a chip 9:2145–2152.

29. Persat A, Marshall LA, Santiago JG (2009) Purification of nucleic acids from whole blood using isotachophoresis. Analytical chemistry 81:9507–9511.

30. Persat A, Santiago JG (2011) MicroRNA profiling by simultaneous selective isotachophoresis and hybridisation with molecular beacons. Analytical chemistry 83:2310–2316.

31. Rogacs A, Qu Y, Santiago JG (2012) Bacterial RNA extraction and purification from whole human blood using isotachophoresis. Analytical chemistry 84:5858–5863.

32. Garcia-Schwarz G, Santiago JG (2012) Integration of On-Chip Isotachophoresis and Functionalised Hydrogels for Enhanced-Sensitivity Nucleic Acid Detection. Analytical chemistry 84:6366–6369.

3. Isotachophoretic fluorescence *in situ* hybridisation of intact bacterial cells

Declarations: This chapter has been submitted as a research article to Analytical Chemistry journal. All effort was made to maintain the original features of this article except minor changes such as the layout, numbering and font size were carried in order to maintain a consistent formatting style of this thesis. Additional information that was not included for the research article has been include in this chapter.

3.1 Abstract

A counter-pressure assisted capillary isotachophoresis method in combiation with sieving matrix and ionic spacer was used to perform in-line fluorescence *in situ* hybridisation (FISH) of bacterial cells. A highly concentrated sieving matrix was introduced at one end of the capillary and the bacterial cells was suspended in the spacer electrolyte for injection. Using a 2 min injection time with 18 psi counter-pressure, 50% of the injected cells that was in the capillary was hybridised with the fluorescently labelled oligonucleotide and the excess unhybridised probe was separated from the hybridised probe in a two stage analysis using single ITP method. The total analysis time reduced to 30 min (9 min run time). This method can also be used for specific detection of bacterial cells in a mixture suspension. The LOD of the in-line hybridisation cells shown to be similar to the existing reported FISH-CE method.

3.2 Introduction

Rapid and robust alternative methods for enumeration of pathogenic microorganisms have evolved significantly over the last decade. These include the use of immunological and molecular techniques as an alternative to the classical standard reference method – the plate count method [1]. This method is used for the detection of bacteria by regulatory agencies and all new methods have to be validated against this [1-4]. Plate count is a low cost method

and provides exquisite sensitivity, being able to quantify 4 colony forming unit per mL (CFU/mL) and 1 CFU/25 g of solid material [1,2]. However, it is time consuming with 24-48 hr of incubation time, the process is laborious, requires significant volumes of broth or agar and it is difficult to automate [1, 5]. Alternate methods, such as immunological techniques, lack the sensitivity at low cell concentrations and enrichment is required prior to analysis [1, 6]. Polymerase chain reaction (PCR) can theoretically detect a single bacteria in the sample, but inhibitors may be present in the sample matrix and poor purity of the target nucleic acids may cause false negatives [1, 6, 7]. Despite this issue, nucleic acid-based methods have allowed detection of cells based on the DNA/RNA sequences without enrichment.

Fluorescence *in situ* hybridisation (FISH) is a molecular technique that is widely used for microbial identification, quantification and, in combination with other techniques, for characterisation of phylogenetically defined microbial populations. Delong *et al.* reported this method for phylogenietic identification of single microbial cells [8]. Subsequently, in 1990, Amann *et al.* used the method in combination with flow cytometry to analyse a mixed microbial population [9]. The advantages of FISH include the ability to visualise whole cells while accurately identifying them by targeting the high abundance ribosomal RNA (rRNA) within the bacterial cells [1, 2, 8-10]. Consequently, in contrast to plate count methods, which rely on bacterial growth, this method detects both non-viable and non-culturable (VBNC) pathogens [2], with a reported detection limit of 10^2 cells/mL for drinking water [4, 11, 12]. However, as discussed by Rohde, a detection limit between 10^3-10^4 cells/mL is more routinely obtained because at least one cell is required per field of view to provide unambiguous results accounting for different losses during sample preparation or statistical variations [2]. The target rRNA used in FISH method are usually either the 16S rRNA of the small ribosomal subunit or 23S rRNA of the large ribosomal subunit [10], allowing FISH to be a promising tool for the replacement of the classical enumeration of bacterial cells.

Although FISH is rapid in comparison to standard plate count method, the FISH protocol before quantification – fixing and staining – takes about two hours. Rapid hybridisation of nucleic acids in free solution was reported by the Santiago's group using

isotachophoresis (ITP) with a sequence specific hybridisation probe, reporting a 960-fold and 14 000-fold increase in the rate of hybridisation [13]. ITP enables concentrating both of the target and probe nucleic acids in a very small volume at the ITP interface. Their initial work using a molecular beacon [13, 14], which increases fluorescence upon hybridisation, was followed by demonstrating hybridisation using a permanent fluorophore [15]. In this later work, a sieving matrix and spacer ion were used to separate the unhybridised excess probe from the hybridised nucleotide after the initial ITP acceleration phase [15].

This chapter examines the ITP accelerated hybridisation approach for FISH staining of intact bacteria. Counter-pressure assisted ITP method is used for hybridisation, followed by the separation of the unhybridised probe from the hybridised cells-probe complexes using a sieving matrix and appropriate spacer ions after an initial ITP acceleration phase. Using this new approach, we demonstrated an in-line FISH staining efficiency of 50%, within 30 min, compared to the off-line staining approach, which required 2.5 hr. Moreover, the in-line FISH staining protocol is suitable for automation, further increasing its potential to be employed as a routine analysis of cells enumeration.

3.3 Methods and Materials

3.3.1 Chemicals

Tris(hydroxymethyl)aminomethane) (Tris), ≥ 99.95%, hydroxyl ethyl cellulose (HEC) (M_w 250 000), polypyrolidine (PVP), (M_w 1300 kDA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 99.5%, 2-(N-Morpholino)ethanesulfonic acid hydrate, magnesium chloride anhydrous ($MgCl_2$), 4-Morpholineethanesulfonic acid (MES) ≥99.5%, dimethyl sulfoxide anhydrous (DMSO) ≥ 99.9%, 1 M Tris HCl (pH 8.0), Coumarin 344 were purchased from Sigma Aldrich (St. Louis, USA). Luria Bertani media (LB, 1 L LB – 10 g tryptone, 5 g yeast extract, 10 g NaCl) , LB agar plates were prepared in house. Tryptone, yeast extract and Phosphate Buffered Saline (PBS) were purchased from Oxoid (Hampshire, England). Sodium chloride was purchased from Univar (Sevenhill, Australia). Agar was purchased from Gelita (Beaudesert, Australia).

3.3.2 Electrolytes

For all ITP experiments, the leading electrolyte (LE) used was 50 mM Tris HCl (pH8.0) with 0.05% HEC w/v, 5 mM $MgCl_2$ and 0.1% v/v DMSO. The terminating electrolyte (TE) was 50 mM Tris HEPES (pH 8.0) with 0.1% v/v DMSO. The spacer electrolyte (SE) used was 50 mM MES adjusted with Tris to pH7.8 with 0.1%v/v DMSO. All solutions were prepared using 18 MΩ Milli-Q water (Millipore, Bedford, MA, USA). The pipettes used were sprayed with Rnase to remove contaminations. Pipette tips used were purchased from Molecular Bioproduct with aerosol resistant tips where the tips are pre-sterilised before packaging. The microtubes and electrolyte solutions used were autoclaved prior using. For plating, aseptic technique was used.

3.3.3 Bacteria growth

The model bacteria, *Escherichia coli (E. coli)* strain TOP10 was obtained from Invitrogen (Mulgrave, Australia) and *Pseudomonas aeruginosa (P. aeruginosa)* strain was obtained in house from the microbiology culture collection (School of Land and Food, University of

Tasmania) were used as model studies. The bacterial cells were grown in solid LB media at 37°C and, when necessary, in liquid LB broth at 37°C with shaking. To obtain *E. coli* and *P. aeruginosa* cells for ITP experiments, a colony from a stock on each of the *E. coli* and *P. aeruginosa* respectively culture were inoculated in LB broth and incubated overnight at 37 °C with shaking. To harvest the cells, 10 mL of the overnight cell culture was transferred to a sterile 15 mL centrifuge tube and centrifuged at for 5 min at 3600 rpm at room temperature (model: Universal 16 A, Hettich, Zentrifugen, Tuttlingen, Germany). The supernatant was carefully discarded and cell pellet was resuspended in sterile PBS. This suspension was stored at 4°C until required and remained viable for up to one week. Cell number of *E. coli* and *P. aeruginosa* colony forming units (CFU equivalent to cells), was determined by plate count enumeration method.

3.3.4 Sample preparations

Neutral dye: 2.6 µM of coumarin 334 is suspended into LE solution and 2.6 µM of *Enterobacteriaceae* probe was suspended into SE solution.

Bacterial cells: 100-500 µL of bacterial cells from the PBS stock was centrifuged for 5 min at 3600 rpm at room temperature (Model: Eppendorf 5424, Hamburg, Germany), the supernatant was discarded and 100 µL of 50 mM SE was added and mixed by gentle vortex for 20 s. This washing step was repeated once. Final volume for analysis of the cells was 500 µL in SE solution with 0.1% v/v DMSO.

3.3.5 DNA probes and hybridisation

Two DNA probes targeting the 16S rRNA of bacterial cells were used which are *Enterobacteriaceae* specific: 5’ TGC TCT CGC GAG GTC GCT TCT CTT 3’ [16] and *Psuedomonas aeruginosa (P. aeruginosa)* (PseaerA) sequence probe: 5’ GGT AAC CGT CCC CCT TGC 3’ [17]. The 3’ end of the *Enterobacteriaceae* probe was labelled with 6 – carboxyfluorescien (FAM) and the 3’ end of PseaerA probe was labelled with Cyanine dye (Cy5). All probes were custom synthesised with HPLC-purification by Intergrated DNA Technology (Coralville, IA) After receipt, the probes were resuspended in Nuclease Free

water at final concentration of 100 μM and stored at – 80 °C. A secondary stock was prepared from the main stock and suspended in the LE solution at final concentration of 26 μM.

For analysis: secondary stock was diluted with the LE solutions with final concentration of the probe to be 2.6 μM.

3.3.6 Capillary Electrophoresis

All CE experiments were carried out on a Beckman Coulter P/ACE MPQ Capillary Electrophoresis System equipped with 488-nm laser module. An external bench top fiber coupled laser with 635 nm wavelength (Thorlabs, New Jerseys, USA) was connected to the second laser port on Beckman Coulter. Experiments were conducted using a bare fused silica capillary (Polymicro Technology, AZ, USA) of 50 μm id with total length of 40 cm (effective length to detector, 30cm). The capillary was maintained at a constant 25°C. Data was collected and analysed using the 32 Karat software version 8.

3.3.7 Capillary Conditioning and isotachophoresis

A new capillary was pre-conditioned at 20 psi in the following order: 1M NaOH (30 mins), milli-Q water (20 mins), 1M HCl (20 min), Milli-Q water (10 min) followed by 1 % w/v PVP at 45 psi for 45 min. Finally, the capillary was conditioned with 50 mM Tris HCl (pH8.0) + 5 mM $MgCl_2$ + 0.05% w/v HEC at – 16 kV for 10 min.

Each analysis began by flushing with 1.8% w/v HEC for 8 min at 75 psi, 2.6μM probe in LE solution for 9 min at 10 psi, EKI injection of sample in SE solution at -16 kV (400 V/cm) with counter pressure of 18 psi for 2 min, followed by EKI injection of TE solution at – 16kV, (400 V/cm).

3.4 Results and Discussions

3.4.1 Schematic of coumarin 334 study of sieving matrix using leader and spacer electrolyte to determine the ITP boundary

Previously, we have demonstrated the ability to quantitate cells down to 135 cells/mL (without counter-pressure) [18] and 78 cells/mL (with counter-pressure) [chapter 2] [19] using a generic intercalation dye (SYTO 9) and ITP. A major limitation of this approach is the lack of specificity as all cells are stained and quantified. Specificity can be introduced through the use of sequence-specific hybridisation probes, as demonstrated initially by Armstrong *et al.* [20] although at the expense of sensitivity, with a detection limit of 1.9 x 10^4 cells/mL. The Santiago's group have recently shown in-capillary/chip hybridisation using ITP [14, 21-24], with 10^3 – 10^4 improvements in hybridisation kinetics. The premise of this work is to evaluate ITP for the rapid specific staining of intact cells. Specifically it is to (i) focus both bacterial cells and FISH probe in a single ITP boundary for rapid hybridisation and (ii) to separate the unhybridised FISH probe from the stained cells into two distinct ITP peaks for quantitation of the cells peak. This requires the establishment of two ITP boundaries created by using the leading ion, terminating ion and an ITP spacer. Separation of the bacterial cells from the unhybridised probe was achieved based on size using a sieving matrix. A sieving matrix of 1.8% HEC was selected, and based on this the leader (chloride), terminator (HEPES) and spacer (MES) selected. This was done on the basis that the bacterial cells and unhybridised probe had a mobility greater than the spacer and less than the leader when there was no sieving matrix present to allow rapid hybridisation, and when entering the sieving matrix region, the bacteria had a mobility lower than the spacer and higher than the terminator and the unhybridised probe was higher than the spacer and lower than the leader.

In order to provide sufficient time to concentrate the cells and probe in the first ITP boundary prior to reaching the sieving matrix, a counter-pressure [19] was used to counter the movement of the ITP boundary. The minimum target was to ensure that at least 10 % of

the total capillary length was without the sieving matrix providing sufficient time and space for the hybridisation to occur. Experiments were conducted using the neutral fluorescent dye coumarin 334 to mark the non-sieving matrix zone and the position of the hybridisation probe monitored to optimised the counter-pressure assisted ITP method.

This section and discussion of figure 3.1A-D are not included in the publication.

Figure 3.1A shows the schematic of the injection of the sieving matrix and coumarin 334 in LE solution, to estimate the total length of the probe in the capillary. Initially, the capillary is filled with 1.8% HEC (figure 3.1A (i)). Using 12 psi pressure, the coumarin 334 in LE solution was injected at different time (figure 3.1A (ii)). After that, the inlet was changed to SE solution and 10 psi pressure was used to push the plug out (figure 3.1A (iii)). From this study, the plug length of coumarin 334 represents the LE with oligonucleotide probes in the capillary in free solution for ITP. The total plug length of coumarin 334 in the capillary was calculated based on the end time minus the start time of the plug shown inset of figure 3.1B. From graph 3.1B, the plug length of coumarin 334 injected corresponded to the injection time (n=3). As the injection time increased, the length of the plug increased. At 10 mins of injection time, when the inlet was changed to SE, the coumarin 334 plug has reaches the detection window (result not show - as the plug length could not be calculated). Thus, at 9 mins of injection, the plug length filled almost 100% of the capillary, pushing out the 1.8% HEC sieving matrix to outlet.

From the injection plug experiments, 4 mins of injection time was selected as the injection time. The next step of the experiment is to ensure in the free solution, the hybridisation between the probe and the bacterial cells falls within the sieving-matrix free zone. Thus, using coumarin 334, the ITP band of the probe in SE should fall within the injection plug. Various counter-pressure was applied during the ITP with EKI (- 16 kV) with different injection time (figure 3.1C (i-v)). From the isotachopherogram in figure 3.1C (i-v), only injection time of 1 min with counter-pressure from (0-18 psi), the ITP bands stay in the coumarin 334 plug (figure 3.1C (i)). When the injection time increases (2-5 min), the probe does not stay in the coumarin plug, instead, the ITP band moves along the capillary during

ITP with various counter-pressure applied (isotachopherogram in (figure 3.1C (ii-v). Morever, the maximum counter-pressure can be applied on the Beckman instrument was 18 psi. To overcome this issue, slight modification was applied.

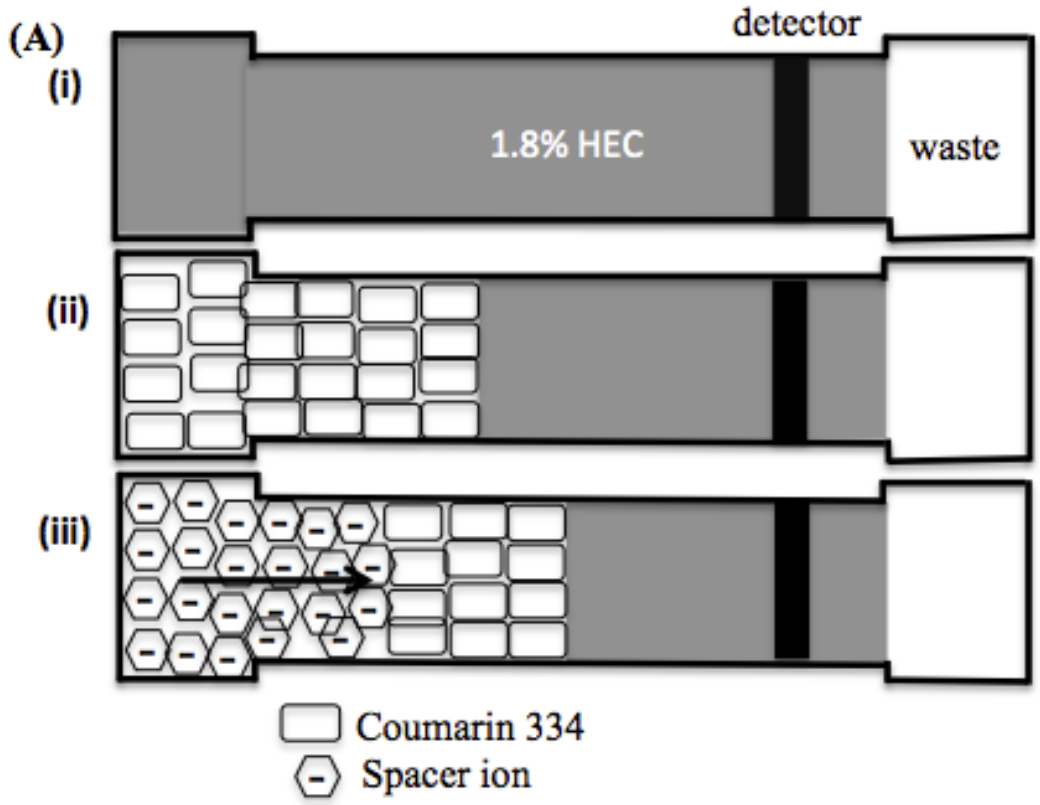

Figure 3.1: (A) Schematic of the coumarin 334 plug analysis. (i): 1.8% HEC sieving matrix was flushed to filled into the whole capillary. (ii) the inlet is changed to coumarin 334 in LE solution. Forward pressure of 12 psi was used to inject the plug into the capillary with different injection time. (iii) the inlet was changed to SE solution, forward pressure of 10 psi was used to push the injected coumarin 334 plug in the capillary to detector.

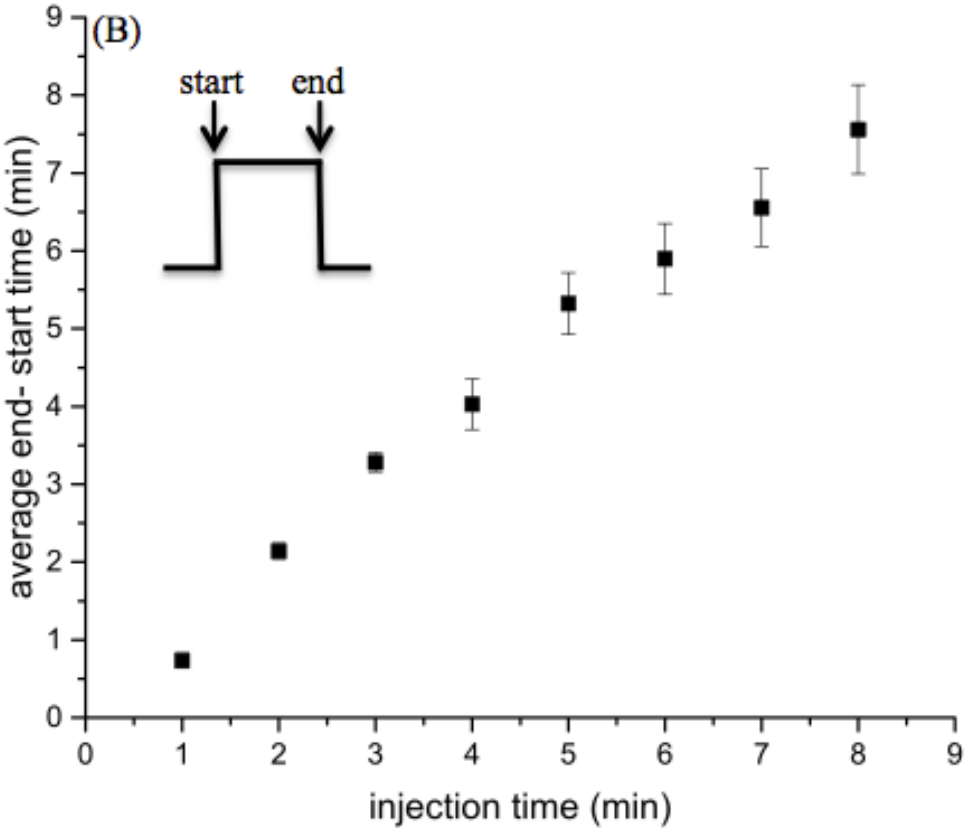

Figure 3.1: (B) Is the graph of the average end-start time of the coumarin 334 plug vs injection time.

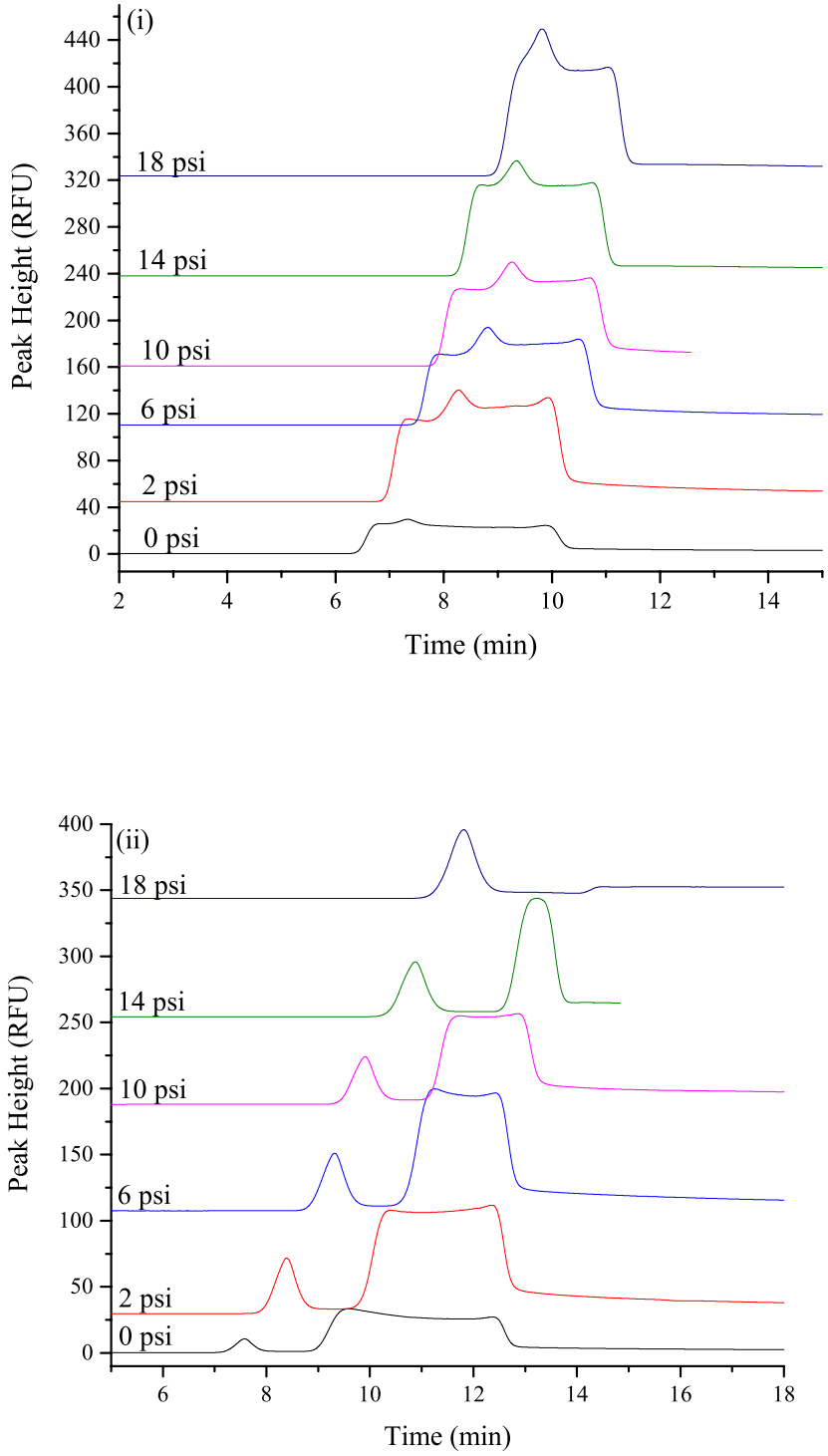

Figure 3.1: (C) (i) Isotachopherogram of coumarin 334 plug with DNA probe with 1 min injection in various counter-pressure applied (0-18 psi). (ii). Isotachopherogram of coumarin 334 plug with DNA probe with 2 min injection in various counter-pressure applied (0-18 psi).

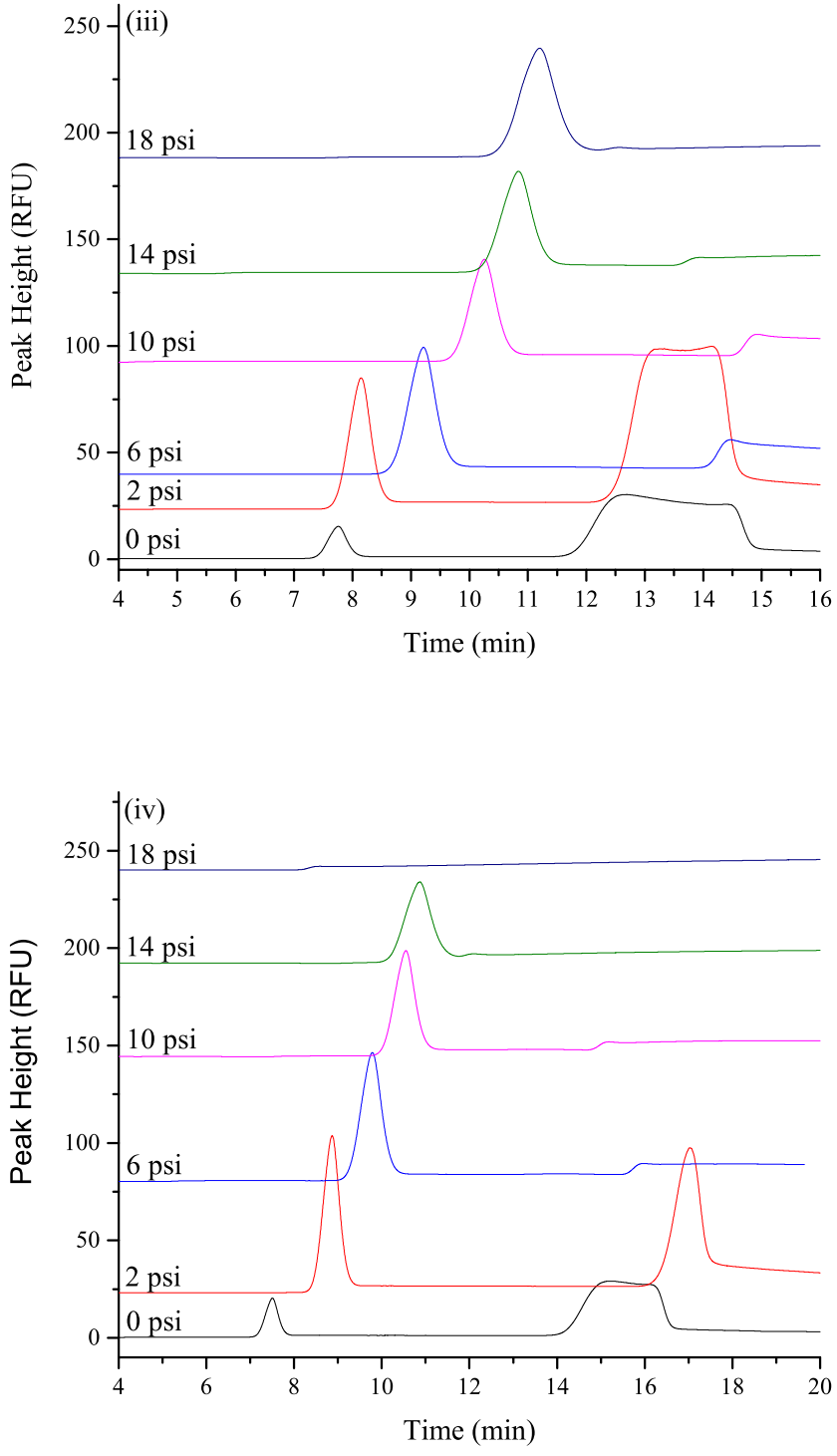

Figure 3.1: (C) (iii). Isotachopherogram of coumarin 334 plug with DNA probe with 3 min injection in various counter-pressure applied (0-18 psi).(iv). Isotachopherogram of coumarin 334 plug with DNA probe with 4 min injection in various counter-pressure applied (0-18 psi).

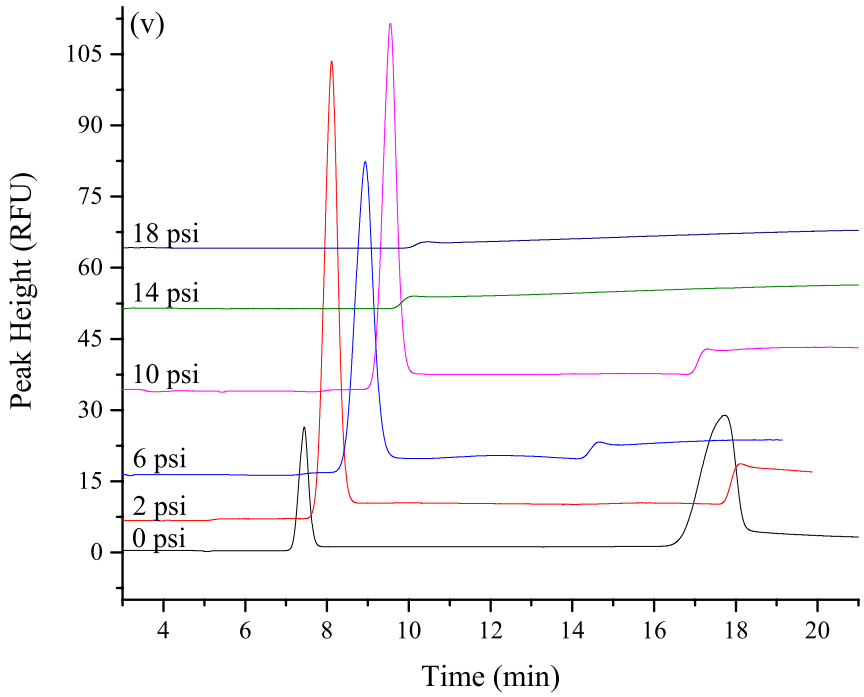

Figure 3.1: (C) (v) Isotachopherogram of coumarin 334 plug with DNA probe with 5 min injection in various counter-pressure applied (0-18 psi). Experimental condition: 50 mM Tris HCl (pH8.0) + 1.8% HEC was flushed at 65 psi for 8 min. Then, inlet is changed to LE solution consist of 50 mM Tris HCl (pH 8.0) + 0.1% v/v DMSO + 5 mM MgCl + 2.6 μM Coumarin 334 flused at 12 psi for 4 min. Inlet changed to SE solution consist of 50 mM MES 75 mM Tris + 2.6 μM *Enterobacteriaceae* probe. Voltage of – 16 kV was applied in the inlet and at the same time counter pressure (various 0-18 psi) was applied with different injection time (1-5 min) to hold the ITP band stationary in the capillary. After EKI injection, voltage and counter-pressure are removed, inlet changed to TE solution containing 50 mM Tris HEPES (pH7.8) and 10 psi pressure was used to push the plug to detector. Total capillary length is 40 cm with 30 cm to detector.

Figure 3.2A shows the schematic of the counter-pressure ITP using coumarin 334 with counter-pressure of 18 psi used. Firstly, the capillary was filled with 1.8% HEC sieving matrix using 65 psi for 9 min (figure 3.2A (i)). The inlet is then changed to LE solution with coumarin 334 (figure 3.2A (ii). At the same time, outlet was filled with 1.8% HEC. The LE solution was flushed at 12 psi for 9 min. However, from figure 3.1B results, we know that an injection time of 9 mins, the sample plug remained in the capillary but at the injection time of 10 min, the sample plug filled 100% of the capillary. After 9 min, the inlet is changed to SE solution with fluorescently labelled oligonucleotide while the outlet remains with 1.8% HEC (figure 3.2A (iii)). When – 16 kV was used to EKI inject the SE and probe into the capillary at various injection time, the counter-pressure inject the 1.8% HEC back into the capillary. During this time, the probes will focus in the ITP band migrating along the plug (figure 3.2A (iv)). After injection for various time (1- 4 min), the counter pressure was removed the inlet is changed to TE solution and outlet is changed to LE solution. A pressure of 10 psi was used to push the plug out (figure 3.2A (v)). Figure 3.2B shows the isotachopherogram of the ITP band of fluorescently labelled probe and the coumarin plug anlysed in 1 to 4 min injection time. From the figure, the ITP band of the fluorescently labelled probe remained in the coumarin 334 plug at 1 and 2 min (black and red) injection time. We postulate that the high viscosity of the 1.8% HEC has impacted the counter-pressure applied at 18 psi to hold the ITP band stationary during the injection causing the ITP band unable to stay stationary in the capillary. Therefore, at injection time of 3 and 4 mins, the ITP band of the probe exit the coumarin 334 plug (figure 3.2- blue and pink). Moreover, due to the instrument issue where higher counter-pressure above 18 psi is not possible, injection time of 2 min is the maximum injection time to ensure the ITP band stays in the plug.

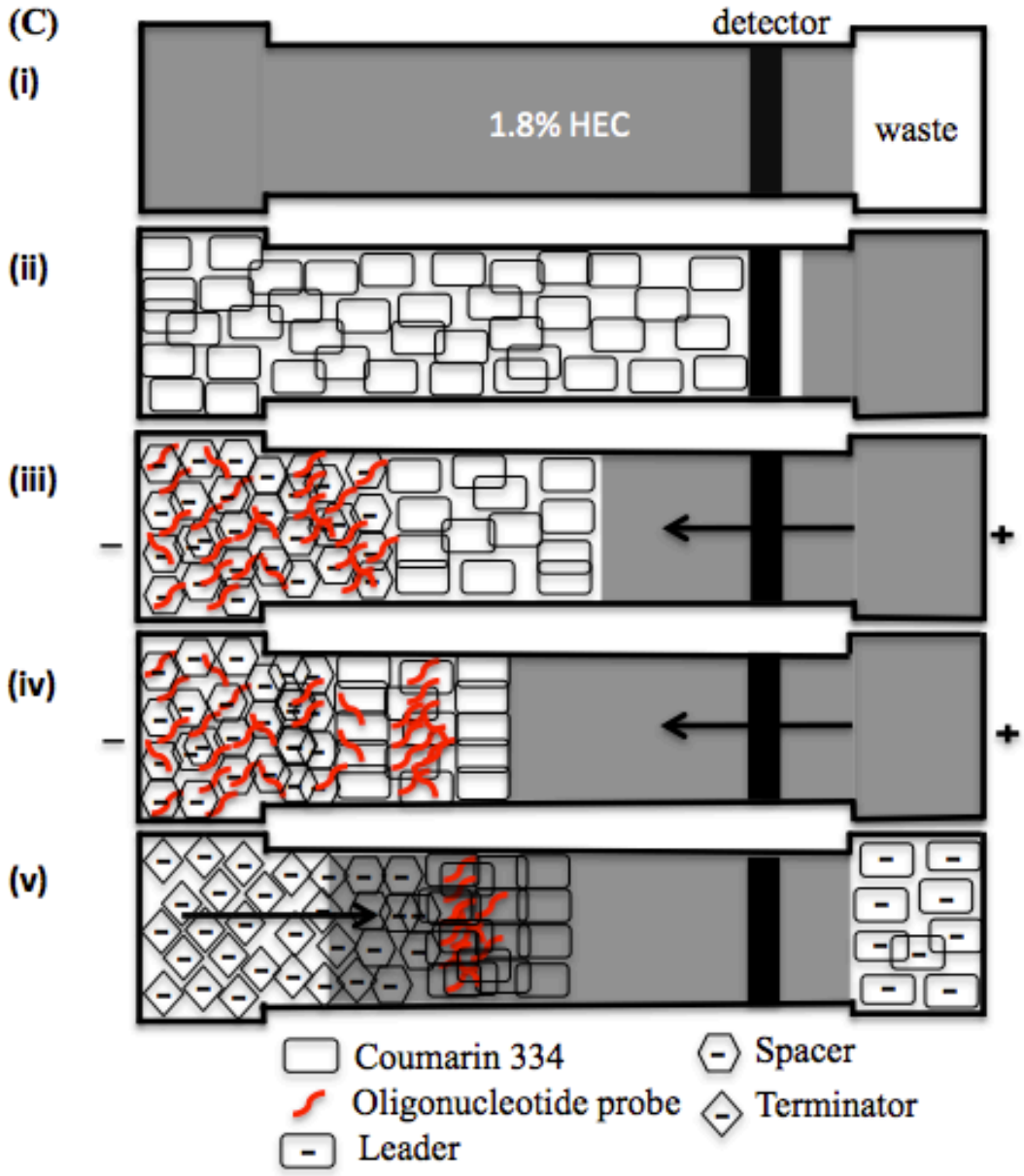

Figure 3.2: (A) Schematic of ITP method using coumarin334. (i); Capillary is flushed with 1.8% HEC sieving matrix. (ii) coumarin 334 in LE is flushed into the capillary at 12 psi for 9 min. (iii) The inlet is changed to SE with 2.6 µM *Enterobacteriaceae* probe. – 16 kV is applied to EKI inject the SE solution into the capillary for various time. At the same time, counter-pressure of 18 psi is applied to inject 1.8% HEC into the capillary from outlet. (iv) Probe are focussed into ITP band within the coumarin 334 plug during injection with Counter-pressure 18 psi. (v) Voltage and pressure are removed, inlet is changed to TE solution, Pressure of 10 psi applied to push the coumarin plug with probe band to detector.

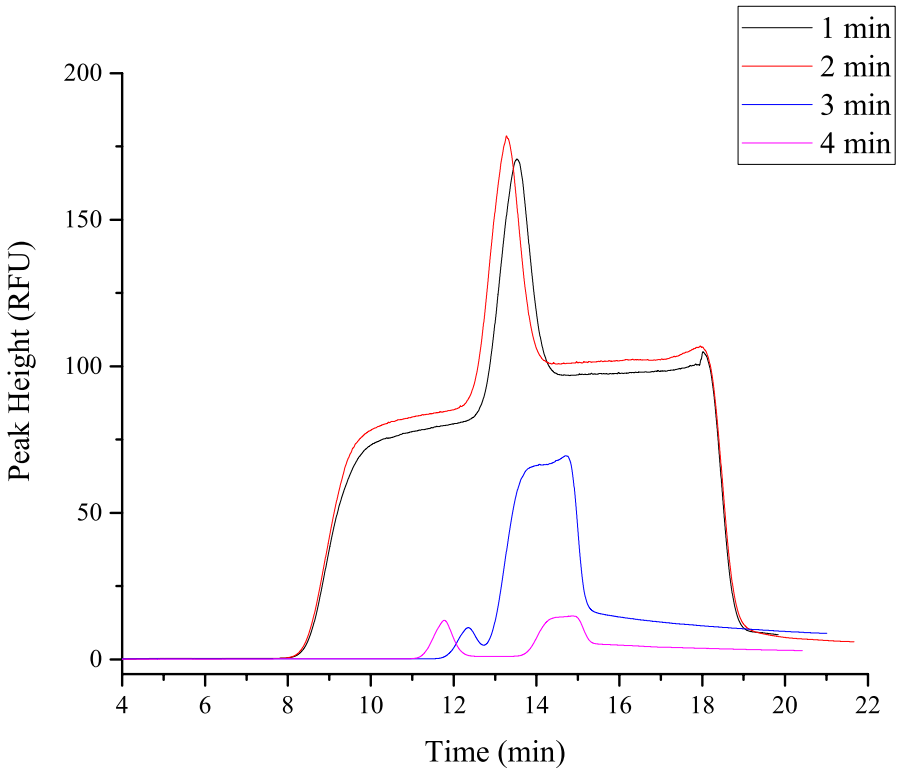

Figure 3.2: (B) Isotachopherogram of the ITP band of probe and coumarin 334 plug with 1 to 4 min injection. Experimental condition: 50 mM Tris HCl (pH8.0) + 1.8% HEC was flushed at 65 psi for 8 min. Then, inlet is changed to LE solution consist of 50 mM Tris HCl (pH 8.0) + 0.1% v/v DMSO + 5 mM MgCl + 2.6 µM coumarin 334 flused at 12 psi for 9 min. Inlet changed to SE solution consist of 50 mM MES 75 mM Tris + 2.6 µM *Enterobacteriaceae* probe. Voltage of – 16 kV was applied in the inlet and at the same time counter pressure (various 0-18 psi) was applied with different injection time (1-4 min) to hold the ITP band stationary and also to flush the 1.8% HEC sieving matrix back into the capillary. After EKI injection, voltage and counter-pressure are removed, inlet changed to TE solution containing 50 mM Tris HEPES (pH7.8) and 10 psi pressure was used to push the plug to detector. Total capillary length is 40 cm with 30 cm to detector.

3.4.2 In-line ITP FISH staining of bacterial cells

From the study above, the optimum procedure is depicted in figure 3.3. First, the capillary was filled with 1.8% HEC. A plug of leading electrolyte with 2.6 μM of the FISH probe was injected into the capillary for 9 min at 12 psi. The inlet was changed to the sample vial where the bacteria were suspended in the spacer ion solution. A voltage of -16 kV and a counter-pressure of 18 psi were applied for 2 min to inject the cells into the capillary and begin the ITP hybridisation process. After 2 min, the counter-pressure was removed and the inlet vial was changed to the terminator solution and outlet vial was changed to leader solution without probe. The same voltage of -16 kV was used to perform the ITP separation. As the ITP band entered the sieving matrix region, the spacer ions overtook the hybridised cells causing them to focus at the spacer/TE boundary. The first ITP boundary between the LE and spacer contained the unhybridised FISH probe, while the second contained the focused cells (figure 3.3 (v)).

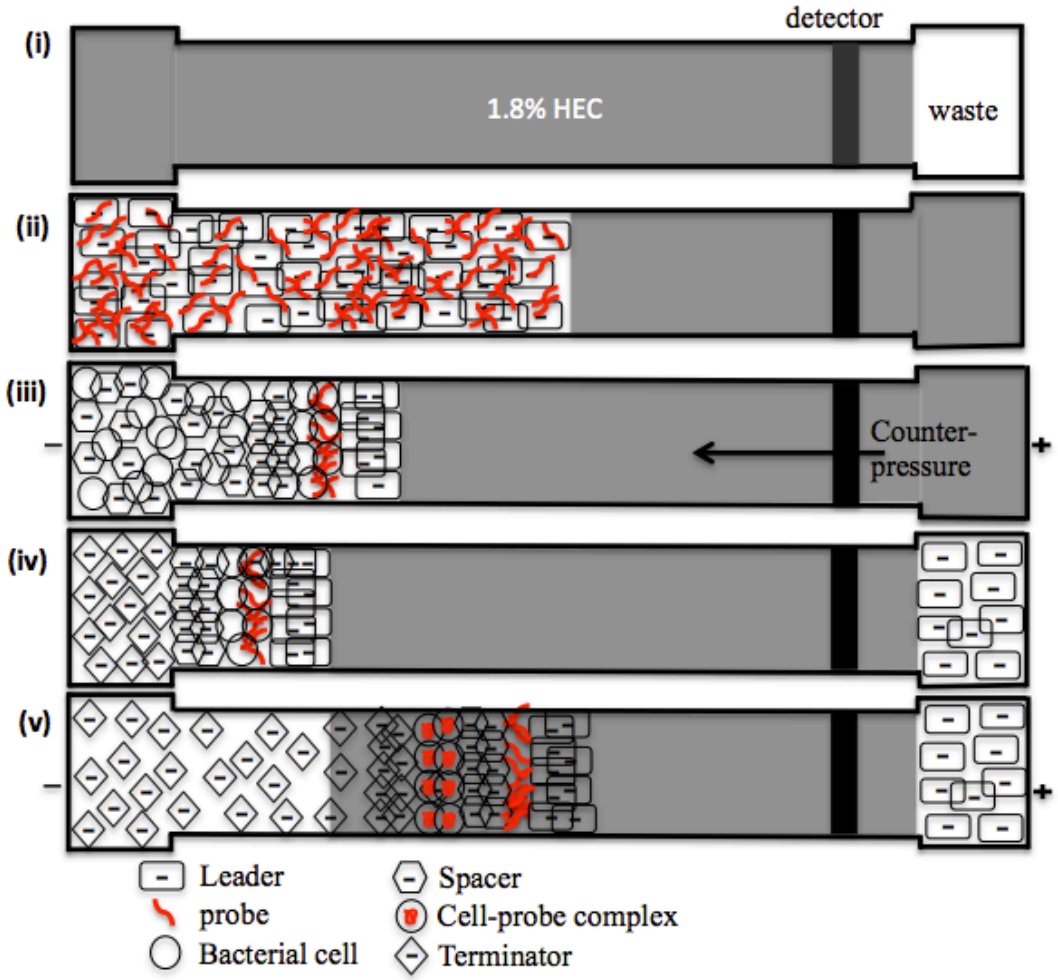

Figure 3.3: Schematic of counter-pressure assisted ITP for In-line fluorescence *in situ* hybridisation. (i) The capillary is filled with 1.8% w/v HEC suspended in 50 mM Tris HCl (pH8.0). (ii) The inlet is switch to LE solutions containing 2.6 µM oligonucleotide with fluorophore and flushed into the capillary at 12 psi for 9 min.(iii) Voltage of – 16kV was applied to EKI the cells in spacer solution into the capillary. At the same time, counter-pressure (18 psi) was applied from the outlet to inject the 1.8% HEC into the capillary while the unhybridised probe and injected cells are focused in the ITP interface for hybridisation. (iv) After 2 mins, the counter-pressure is removed, inlet is changed to TE solution for EKI injection allowing the ITP band to move toward detector. (v) as the ITP band reaches the sieving matrix, the spacer ions will overtake the hybridised probe cells and focussed behind the unhybridised probe band and the cells-probe complex are focused behind the spacer ions but in front of TE solution.

The counter-pressure ITP method from figure 3.3 was evaluated for the analysis of *Escherichia coli (E. coli)* and *Pseudomonas aeruginosa (P. aeruginosa)* with an *Enterobacteriaceae* and PSeaerA probe respectively. Figure 3.4A is the isotachophoregram of different cell concentrations of *E. coli*, while figure 3.4B shows isotachopherograms of different cell concentration of *P. aeruginosa* with PseaerA probe. These results clearly demonstrated in-line staining of the cells with the FISH probe with peaks for the cells that were well separated from the excess unhybridised probe. The inserts in each isotachopherograms showed the linearity plots for both *E. coli* and *P. aeruginosa* cells with good linearity obtained for both. The LOD of *E. coli* calculated to be 6.8×10^7 cells/mL and the LOD for *P. aeruginosa* calculated to be 2.7×10^5 cells/mL based on the calibration curves. The two order magnitude differences between LOD of *E. coli* and *P. aeruginosa* was due to the laser used (635 nm v 488 nm), photobleaching of the fluorophore and/or to differences in the amount of ribosomes in the cells.

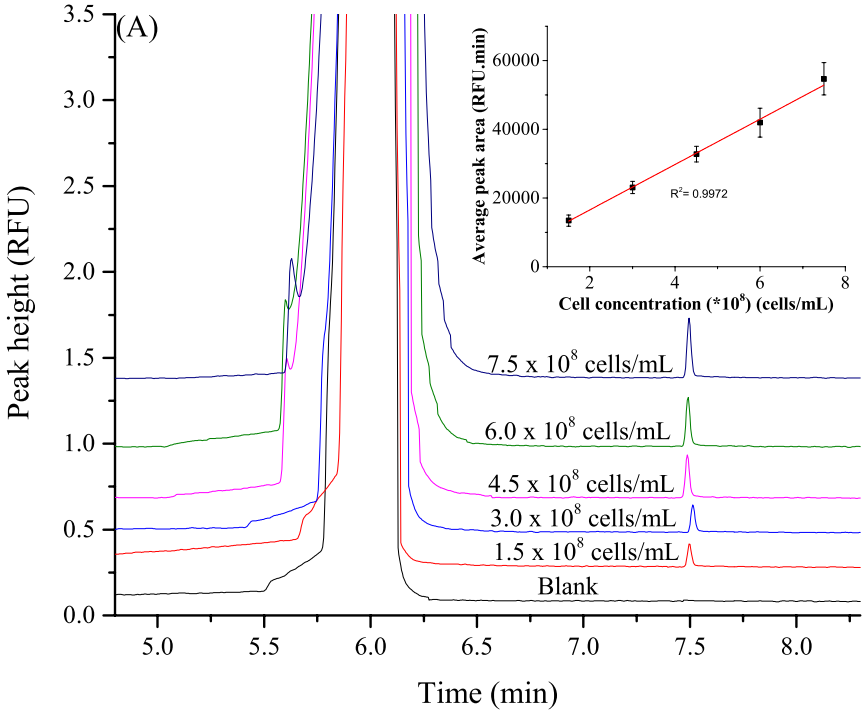

Figure 3.4: (A) Isotachopherogram of *E. coli* analysed with *Enterobacteriaceae* probe at different concentration. Inset is the calibration curve of different *E. coli* concentration vs average peak area (n-=3)

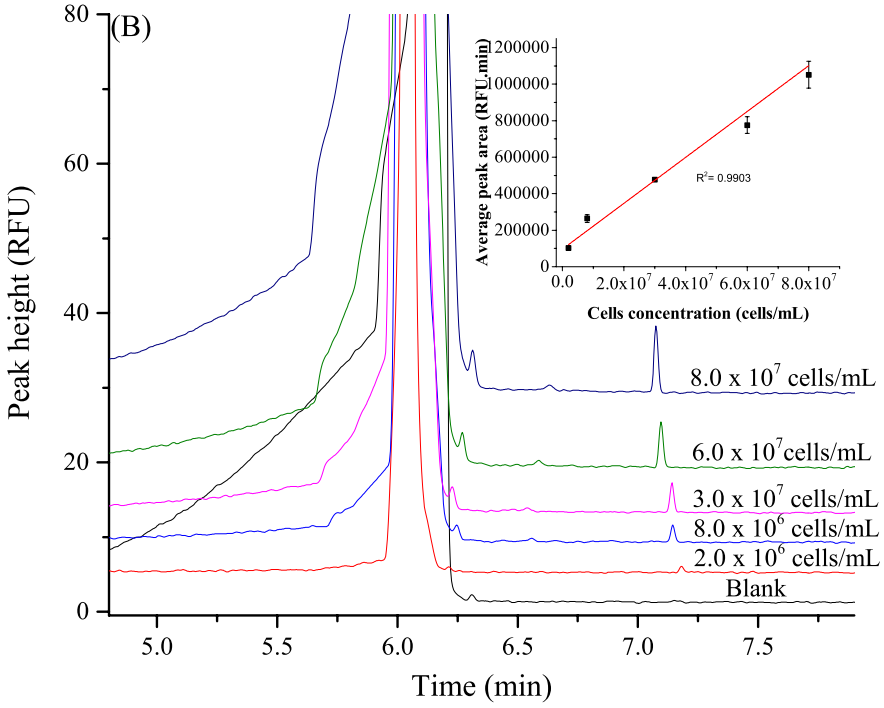

Figure 3.4: (B) Isotachopherogram of *P. aeruginosa* analysed with PseaerA probe at different concentration. Inset is the calibration curve of different *P. aeruginosa* concentration vs average peak area (n-=3). Experimental condition: 50 mM Tris HCl (pH8.0) + 1.8% HEC was flushed at 65 psi for 8 min. Then, inlet is changed to LE solution consist of 50 mM Tris HCl (pH 8.0) + 0.1% v/v DMSO + 5 mM MgCl + 2.6 µM probe flushed at 12 psi for 9 min. Inlet changed to SE solution consist of 50 mM MES 75 mM Tris + cells. Voltage of – 16 kV was applied in the inlet and at the same time counter-pressure (18 psi) was applied for 2 min to hold the ITP band stationary and also to flush the 1.8% HEC sieving matrix back into the capillary. After EKI injection, voltage and counter-pressure are removed, inlet changed to TE solution containing 50 mM Tris HEPES (pH7.8) and voltage of - 16 kV was applied to allow the ITP bands to move towards the detector. Total capillary length is 40 cm with 30 cm to detector.

3.4.3 Specificity of the in-line staining

The probes used above were selected from the literature to evaluate the specificity of the probes and the ITP process. An *Enterobacteriaceae* probe was selected based on the sequence at 1251 – 1274 of the *E. coli* 16S rRNA which is a highly conserved region that allows *in situ* hybridisation of *Enterobacteriaceae* without a false positive reaction [16]. The oligonucleotide was attached with 6-FAM fluorophore allowing detection with excitation at 488 nm. The PseaerA probe was selected because it is reported to specifically bind to *P. aeruginosa* [17] cells and was modified with cy5 flurophore allowing excitation with at 635 nm. To demonstrate selectivity of the probes, experiments were performed using *E. coli* with the PseaerA probe, and *P. aeruginosa* with the *Enterobacteriaceae* probe. The results, shown in figure 3.5(A-B), demonstrate that the *Enterobacteriaceae* probe provides a detectable response with *P. aeruginosa*, but *E. coli* does not provide any response with the PseaerA probe. A nucleotide BLAST check of the *Enterobacteriaceae* probe with 16S rRNA of *P. aeruginosa* identified a 7 bp matches which explains the response of *P. aeruginosa* with this probe. A similar BLAST check was performed with the PseaerA probe and *E. coli* cells with no bp match, indicating no hybridisation would occur between the cells and the probe, demonstrating in-line selective staining of bacteria.

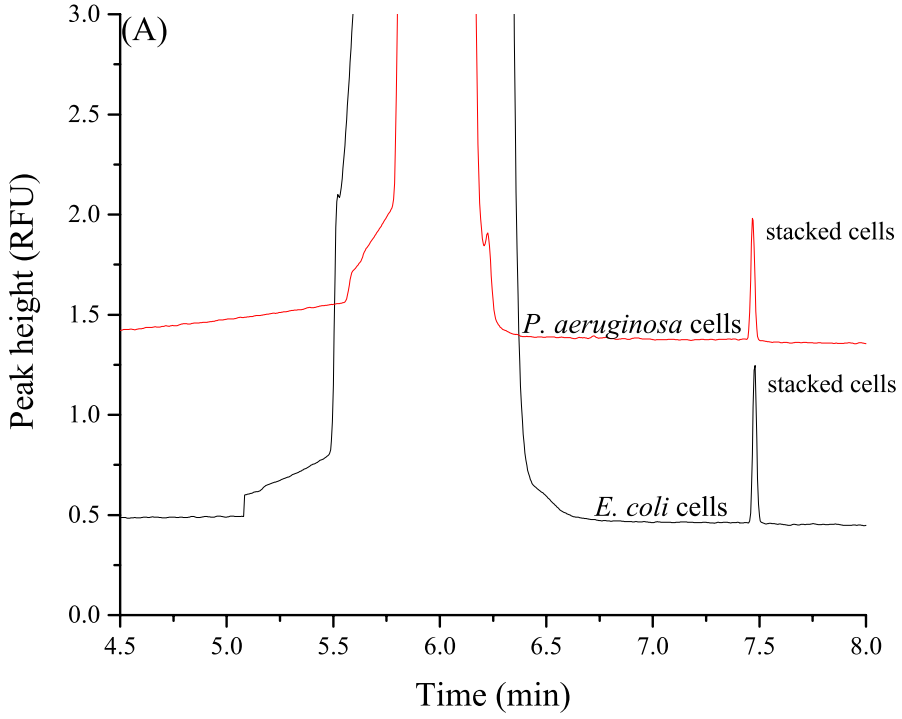

Figure 3.5: (A) Isotachopherogram of *E. coli* and *P. aeruginosa* analysed using 2.6 µM *Enterobacteriaceae* probe.

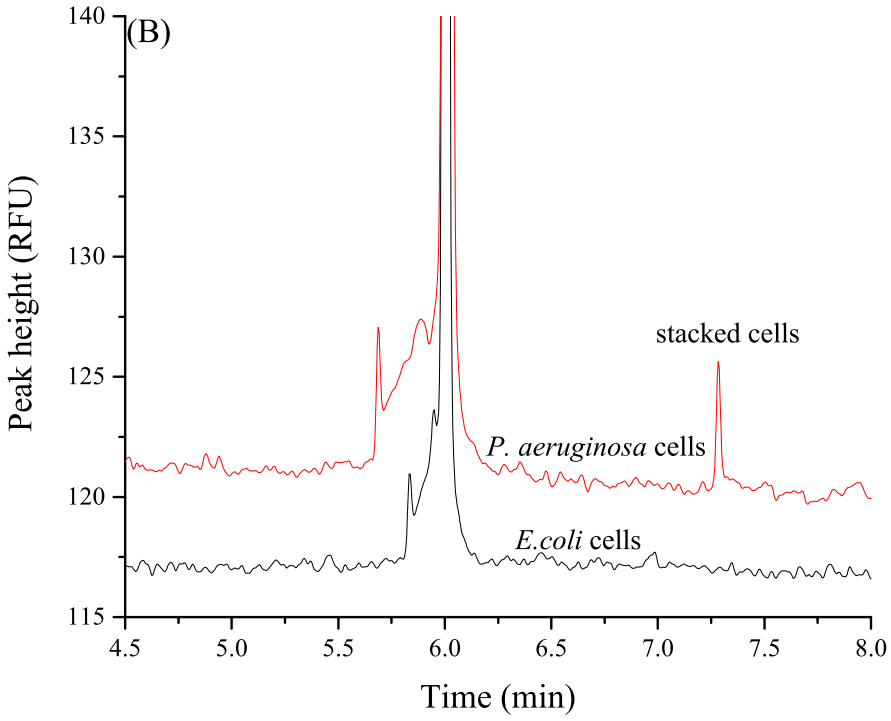

Figure 3.5: (B) Isotachopherogram of *E. coli* and *P. aeruginosa* cells analysed using 2.6 µM of PseaerA probe. Experimental conditions: same as described in figure 3.4

Experiments were then conducted using the PsearA probe with a mixed community of both *E. coli* and *P. aeruginosa*. The ratio of *E. coli* to *P. aeruginosa* cells was varied by keeping the *P. aeruginosa* concentration fixed and increasing the amount of *E. coli*. This was performed with a 2.6 µM of PsearA probe. When the concentration of the *E. coli* increased in the mixture, the signal of *P. aeruginosa* decreased (figure 3.6A). There were two possible reasons: uptake of the hybridisation probe into the *E. coli* cells without binding and/or electrokinetic discrimination.

To examine whether the addition of *E. coli* absorbed the probe, the effect of the concentration of the probe was examined first with a single culture by increasing the concentration of the *P. aeruginosa* selective PseaerA probe while keeping the concentration of *P. aeruginosa* (2.7×10^6 cells/mL) constant. The results are shown in figure 3.6B where it can be seen that as the probe concentration increases, the signal of the second ITP

(hybridised cells) signal increases. The signal increased by 4.7 and 5.3-fold when the probe concentration was increased from 2.6, 26 and 52 μM, respectively. While the highest response was with 52 μM, due to the cost and volume issues of the probe, higher concentrations were not examined, and given the marginal improvement with 52 μM, the lower concentration of 26 μM was used for further evaluation. The mixed culture experiments were repeated with this higher amount of probe. The data in figure 3.6A with the higher concentration of probe shows a higher detector response, but the signal still decreased as the amount of *E. coli* in the sample was increased. By normalising the response to the initial signal without any *E. coli* (figure 3.6C), it can be seen that with the higher concentration of probe used, the relative decrease is less, with approximately 90% of the signal retained when *E. coli* was less than 50 times excess over the amount of *P. aeruginosa*. In higher excess, the signal decreased more rapidly suggesting that there was an influence of the non-target cells on the stained target cell response. The initial slight decrease in response also suggested some influence of electrokinetic bias. The use of hydrodynamic injection is a potential solution to this, but as demonstrated previously, significantly lower detection limits are achieved with electrokinetic injection.

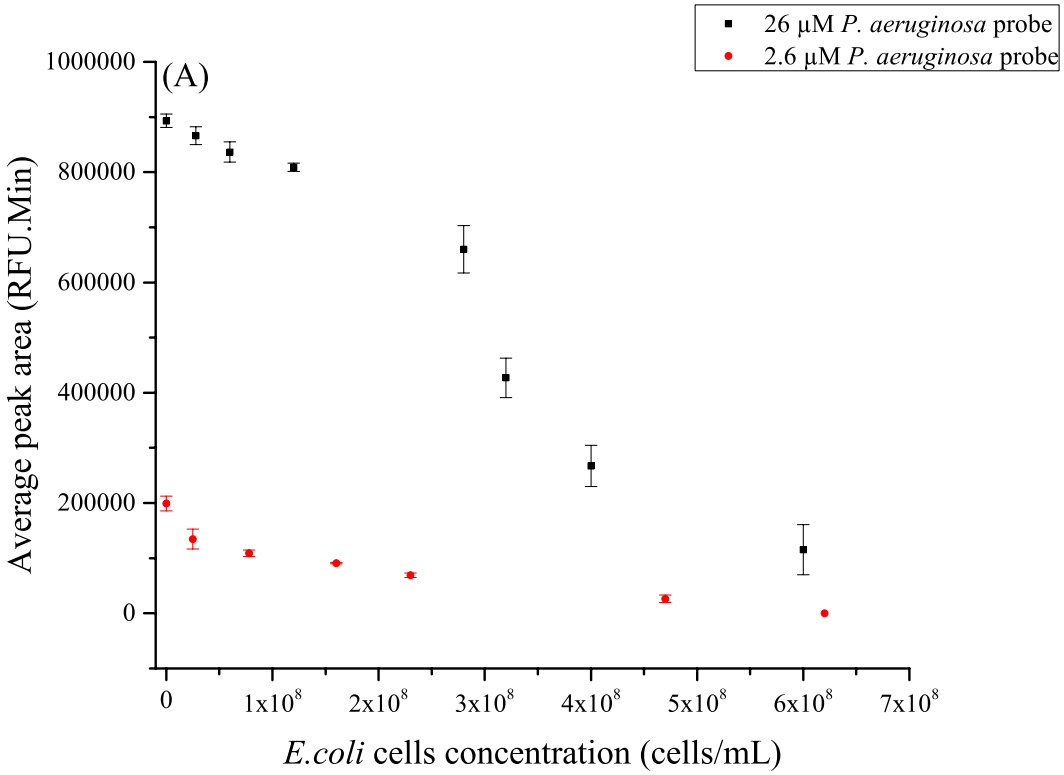

Figure 3.6: (A) Graph of 2.6 µM and 26µM PseaerA concentrations vs different *E. coli* mixture mix with constant *P. aeruginosa* cell concentration.

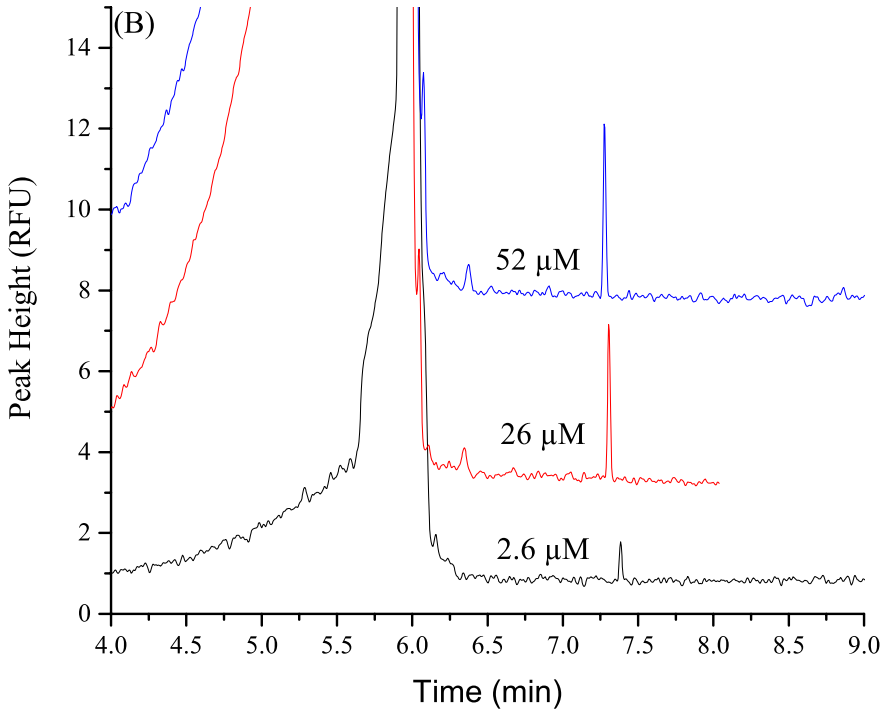

Figure 3.6: (B) Isotachopherogram of different PseaerA probe concentrations using constant *P. aeruginosa* cell concentration (2.7 x 10^6 cells/mL).

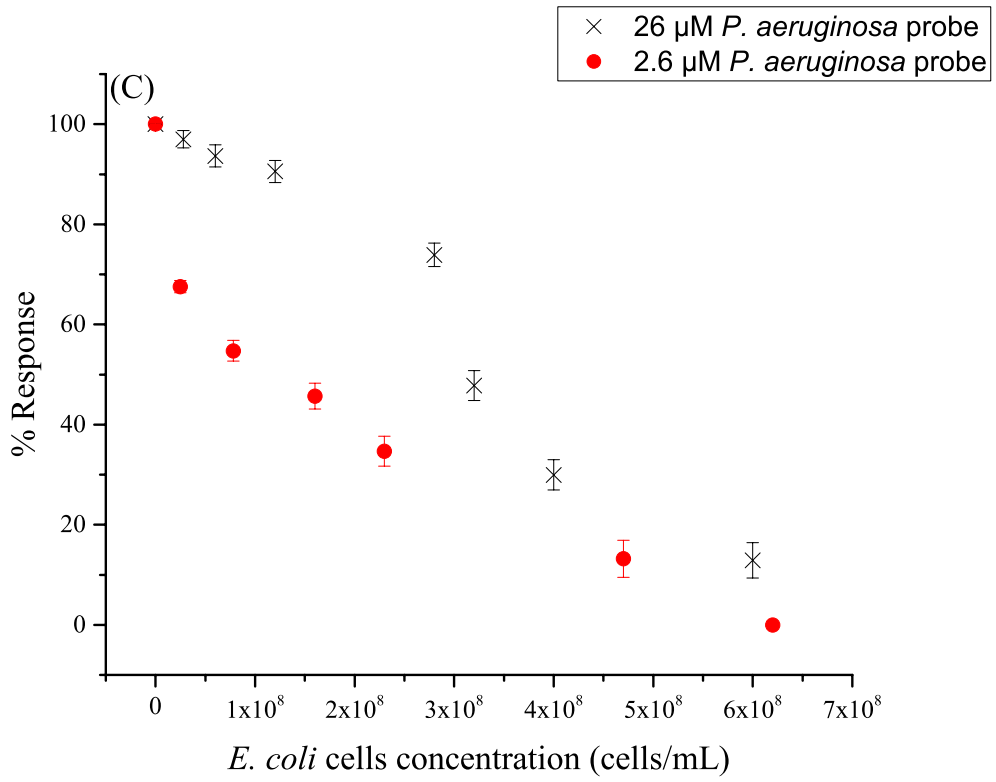

Figure 3.6: (C) Graph response of 2.6 µM and 26µM PseaerA concentrations vs different *E. coli* mixture mix with constant *P. aeruginosa* cell concentration. Experimental conditions: same as describe in figure 3.4 exceptional of the probe concentration in LE and cells concentration in SE as described in results and discussion.

3.4.4 In-line versus Off-line cells staining

To benchmark the performance of the in-line staining process, samples stained off-line using a conventional FISH staining were compared with the ITP approach. A sample of 1000 µL of 1.0 x 10^5 cells/mL of *P. aeruginosa* were fixed with 60:40 Ethanol:PBS based on the procedure described by Lantz *et al.* [20]. The fixed cells were then stained using the FISH protocol from Glöckner *et al.* [25], in solution in an eppendorf tube instead of on a membrane. Once the cells were stained, excess probe was removed using washing buffer,

and the off-line FISH cell was suspended into SE. A sample containing 100 μL of the cells suspension was injected using the counter-pressure ITP method, without probe in the LE. The in-line method used the same number of cells suspended in spacer electrolyte that contained 0.1 v/v of DMSO for permeabilisation of the probe into the cells during ITP. A probe concentration of 26 μM was added to the LE. Representative isotachopherograms are shown in figure 3.7A with the peak for the in-line stained cells, being 50% (n=3) compared to the off-line value. The confirmation for this proportion of staining was done by passing the off-line and in-line stained cells respectively through a black filter for cell counting with a fluorescent microscope. From figure 3.7B, 17 pictures were taken at different positions as showed in the schematic. Based on the area of the filter and the depth field of the microscope, the average of the total cell count of the off-line FISH cells without ITP was 2.24×10^7 cells/mL, from a total of 2.50×10^7, which indicated that the off-line FISH method hybridised ~90% of the total cells in the solution. Collection of the ITP band in a sample vial and collecting on a separate filter yielded 5.32×10^6 cells/mL further verifying that the in-line approach stains approximately 50% of the cells (inset table in figure 3.6B).

The LOD reported by Lantz *et al.* [20] was 1.9×10^4 cells/mL with their CE based method combined with a 30 min off-line FISH staining prior to a 10-15 min electrophoretic analysis. The LOD of this in-line approach is 6.0×10^4 cells/mL, which is approximately 3 times higher, and is predominantly due to the reduced staining efficiency of 50% of the in-line process, together with other differences such as different light sources, PMTs and fluorophores.This reduction of the LOD was confirmed by using the off-line FISH approach, where a concentration of 3.0×10^4 cells/mL were stained and compared to in-line FISH method staining of 6.0×10^4 cells/mL. The isotachopherograms are shown in figure 3.7C.

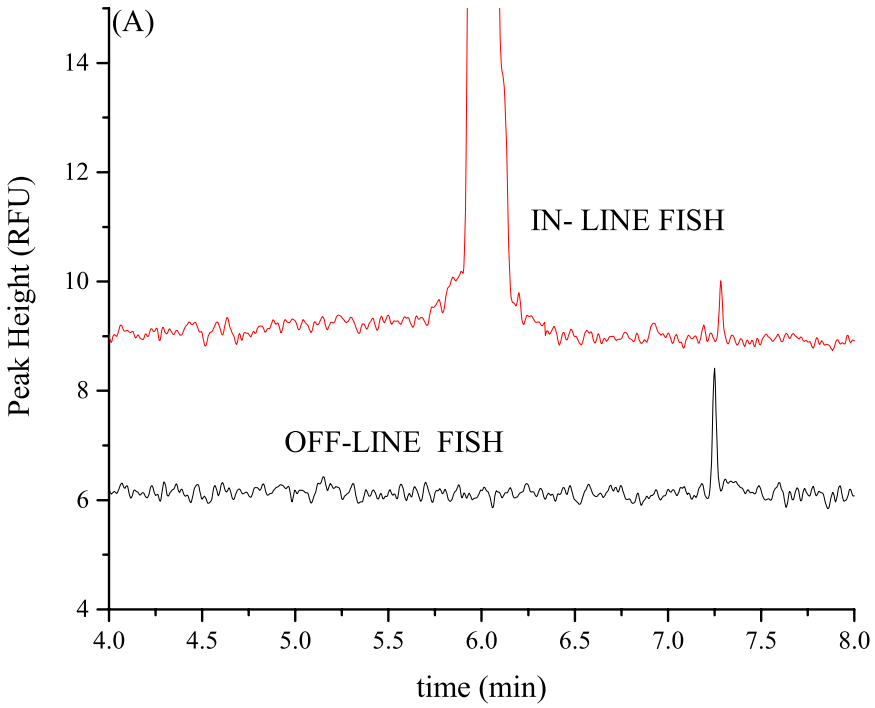

Figure 3.7: (A) Isotachopherogram comparison between in-line FISH ITP of *P. aeruginosa* cells and offline FISH ITP of *P. aeruginosa* cells at constant *P. aeruginosa* concentration.Experimental conditions as described in figure 3.4 with exceptional of 26 µM of probe was used in LE for in-line and no probe was added for off-line.

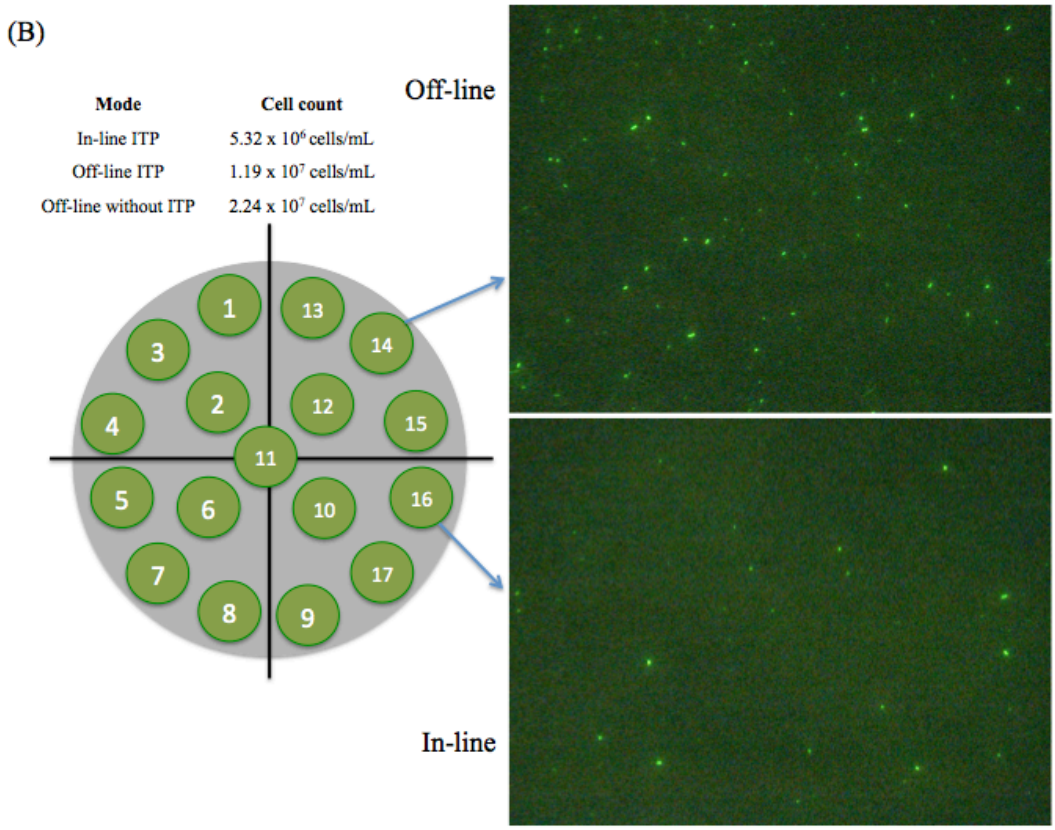

Figure 3.7: (B) image of cell count of cells analysed using counter pressure ITP method for both in-line and off-line with same cell concentration with inset of the total cell counts for each methods.

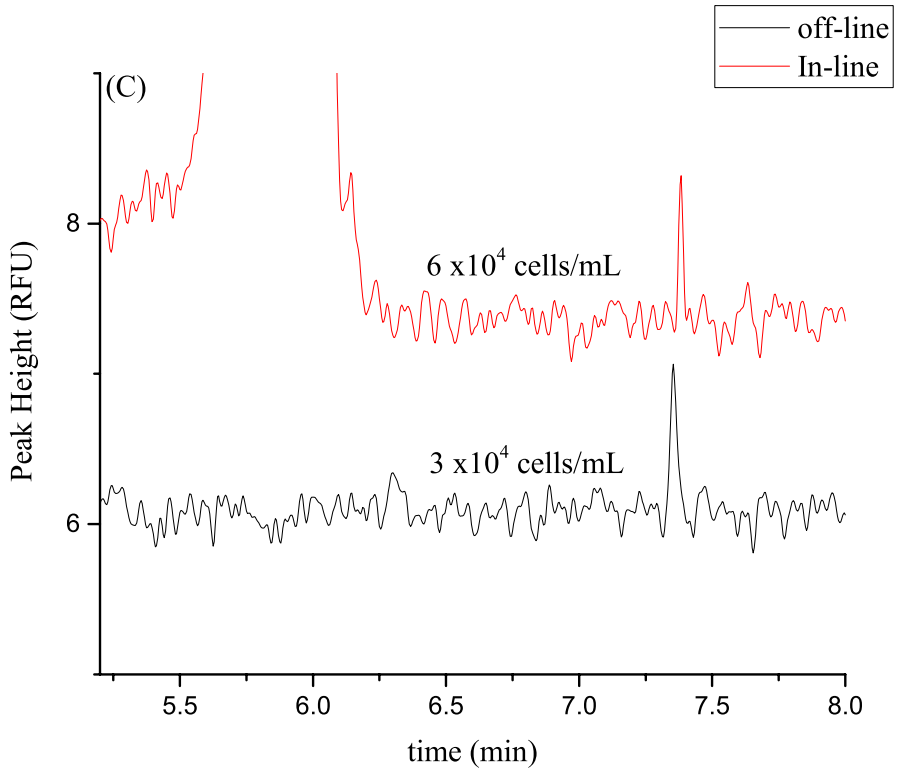

Figure 3.7: (C): Isotachopherogram of 3.0 x 10^4 cells/mL stained off-line FISH method and 6.0 x 10^4 cells/mL stained in-line FISH method using counter pressure ITP.Experimental conditions: as described in figure 3.4 with exceptional the probe concentration used was 26 µM in LE

3.5 Concluding remarks

A dual stage ITP process for the rapid in-line hybridisation and subsequent ITP quantitation of bacteria was developed. This method is able to stain, separate and quantitate bacteria suspensions with minimum sample preparation within 10 min, down to 6×10^4 cells/mL. While the selectivity is achieved through the use of sequence-specific fluorescently labelled oligonucleotides, the presence of other cells within the sample impacts minimally upon detection limits when less than 50 times excess of cells is present, but the signal is compromised at higher ratios due to the consumption of the probe and electrokinetic bias. Further improvements in the type of fluorophore and large volume hydrodynamic injection approaches would overcome this limitation and lead to automated instrumentation for direct quantitation of cells in liquid samples.

3.6 References

1. Jasson V, Jacxsens L, Luning P, Rajkovic A, Uyttendaele M (2010) Alternative microbial methods: An overview and selection criteria. Food microbiology 27:710–730.

2. Rohde A, Hammerl JA, Appel B, Dieckmann R, Dahouk Al S (2015) Food Microbiology. Food microbiology 46:395–407.

3. Roche Y, Cao-Hoang L, Perrier-Cornet J-M, Waché Y (2012) Advanced fluorescence technologies help to resolve long-standing questions about microbial vitality. Biotechnology Journal 7:608–619.

4. Hammes F, Egli T (2010) Cytometric methods for measuring bacteria in water: advantages, pitfalls and applications. Anal Bioanal Chem 397:1083–1095.

5. Souii A, M'hadheb-Gharbi MB, Gharbi J (2016) Nucleic acid-based biotechnologies for food-borne pathogen detection using routine time-intensive culture-based methods and fast molecular diagnostics. Food Sci Biotechnol 25:11–20.

6. Theron J, Eugene Cloete T, de Kwaadsteniet M (2010) Current molecular and emerging nanobiotechnology approaches for the detection of microbial pathogens. Critical Reviews in Microbiology 36:318–339.

7. Barken KB, Haagensen JAJ, Tolker-Nielsen T (2007) Advances in nucleic acid-based diagnostics of bacterial infections. Clinica Chimica Acta 384:1–11.

8. DeLong EF, Wickham GS, Pace NR (1989) Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science 243:1360–1363.

9. Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analysing mixed microbial populations. Applied and Environmental Microbiology 56:1919–1925.

10. Amann R, Fuchs BM (2008) Single-cell identification in microbial communities by improved fluorescence in situ hybridisation techniques. Nat Rev Micro 6:339–348.

11. Hammes F, Berney M, Wang Y, Vital M, Köster O, Egli T (2008) Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes. Water Research 42:269–277.

12. Hammes F, Broger T, Weilenmann H-U, Vital M, Helbing J, Bosshart U, Huber P, Peter Odermatt R, Sonnleitner B (2012) Development and laboratory-scale testing of a fully automated online flow cytometer for drinking water analysis. Cytometry 81A:508–516.

13. Bercovici M, Han CM, Liao JC, Santiago JG (2012) Rapid hybridisation of nucleic acids using isotachophoresis. Proceedings of the National Academy of Sciences of the United States of America 109:11127–11132.

14. Bahga SS, Han CM, Santiago JG (2012) Integration of rapid DNA hybridisation and capillary zone electrophoresis using bidirectional isotachophoresis. Analyst 138:87–90.

15. Eid C, Garcia-Schwarz G, Santiago JG (2013) Isotachophoresis with ionic spacer and two-stage separation for high sensitivity DNA hybridisation assay. Analyst 138:3117–3120.

16. Ootsubo M, Shimizu T, Tanaka R, Sawabe T, Tajima K, Yoshimizu M, Ezura Y, Ezaki T, Oyaizu H (2002) Oligonucleotide probe for detecting Enterobacteriaceae by in situ hybridisation. J Appl Microbiol 93:60–68.

17. Hogardt M, Trebesius K, Geiger AM, Hornef M, Rosenecker J, Heesemann J (2000) Specific and Rapid Detection by Fluorescent In Situ Hybridisation of Bacteria in Clinical Samples Obtained from Cystic Fibrosis Patients. J Clin Microbiol 38:818–825.

18. Phung SC, Nai YH, Powell SM, Macka M, Breadmore MC (2013) Rapid and sensitive microbial analysis by capillary isotachophoresis with continuous electrokinetic injection under field amplified conditions. Electrophoresis 34:1657–1662.

19. Phung SC, Nai YH, Macka M, Powell SM, Guijt RM, Breadmore MC (2015) Counter-pressure-assisted ITP with electrokinetic injection under field-amplified conditions for bacterial analysis. Anal Bioanal Chem 407:6995–7002.

20. Lantz AW, Brehm-Stecher BF, Armstrong DW (2008) Combined capillary electrophoresis and DNA-fluorescence in situ hybridisation for rapid molecular identification of Salmonella Typhimurium in mixed culture. Electrophoresis 29:2477–2484.

21. Persat A, Santiago JG (2011) MicroRNA profiling by simultaneous selective isotachophoresis and hybridisation with molecular beacons. Analytical chemistry 83:2310–2316.

22. Rogacs A, Qu Y, Santiago JG (2012) Bacterial RNA extraction and purification from whole human blood using isotachophoresis. Analytical chemistry 84:5858–5863.

23. Marshall LA, Han CM, Santiago JG (2011) Extraction of DNA from malaria-infected erythrocytes using isotachophoresis. Analytical chemistry 83:9715–9718.

24. Bercovici M, Kaigala GV, Mach KE, Han CM, Liao JC, Santiago JG (2011) Rapid Detection of Urinary Tract Infections Using Isotachophoresis and Molecular Beacons. Analytical chemistry 83:4110–4117.

25. Glöckner FO, Amann R, Alfreider A, Pernthaler J, Psenner R, Trebesius K, Schleifer K-H (1996) Situ Hybridisation Protocol for Detection and Identification of Planktonic Bacteria. Systematic and Applied Microbiology 19:403–406.

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Some segments of the chapter have been published in two conference proceedings, (see p. vi for details).

5. General Conclusions and Future Directions

5.1 General Conclusions

The aim of this research was to develop a method for rapid and robust detection of intact bacterial cells. Isotachophoresis (ITP) was demonstrated to be able to be used as the sole method for *Escherichia coli (E. coli)* quantification using an intercalating dye.

Plate count method is still the standard method for bacteria enumeration due to the capability to detect to cells as low as 4 CFU/mL or 1CFU/25g [1]. The detection limit in using ITP was not as close to the plate count method in terms of sample volume. The first aim of this research was to improve the sensitivity of the ITP method for bacterial cells detection to lower the detection limit. This was reported in chapter 2 where a counter-pressure was implemented into the ITP methods and successfully lowered the detection limit of *E. coli*. The aimed was to keep the ITP band stationary in the capillary for a period of time, to allow more cells in the sample vial to be injected and focussed in the stationary ITP band. The total capillary length to detector indirectly determines the number of cells focussed in the ITP band. Negatively charged encapsulated fluorescent beads with a diameter of 0.51 μm was used for the optimisations study. Various optimisation experiments were conducted to optimise the ITP method including pressure for counter-pressure, injection voltages and injection times. For the very first time, we reported the detection limit of the *E. coli* to be 78 cells/mL, which is the lowest detection limit detected using CE from the time of writing this thesis. In addition, less sample preparation is required for this method where the cells used are live intact cells retrieved from LB broth after overnight incubation at 37 °C, suspended in TE solution with 1μM SYTO 9 and incubated for 30 mins before injection for analysis. The total analysis time was 17 mins. This detection limit achieved was also 4 times lower compared to an identical method without the counter-pressure. The calculated detection limit was also examined by injecting samples of 100 cells/mL (10 cells in 100 μL) and a peak was observed and well distinguished from the blank peak. The % RSD for 100 cells/mL was

calculated to be 7.3% while the % RSD for the blank was 8.5% (n=5). There was no steep response in detection signal at this low numbers of cells. We speculate that this could be because of the intercalation dye used (SYTO 9) which has stained both DNA and RNA inside the cells and the total nucleic acid in the cells vary depending on the growth of the cells. Although this could potentially introduce some inaccuracy in the quantitation at low cells number, the cells at 100 cells/mL in the sample provide a peak area that is acceptable and well distinguished from the blank peak. Moreover, the sample volume used in the study is 100 µL, thus at 100 cells/mL there are only 10 or less cells in the sample volume. The plate count was used to measure the total cell count before injection and after injection without staining the cells with SYTO 9. From the results (n=6), at least 50% of the cells were injected into the capillary for analysis, thus, the peak observed in the isotachopherogram when the sample contained 100 cells/mL represents approximately 5 cells per analysis.

While the counter-pressure assisted ITP method demonstrated success in detecting intact bacterial cells at low concentration with the LOD calculated to be 78 cells/mL, the focus of the second part of the research was to apply the ITP for detection of specific bacteria. SYTO 9 dye used in chapter 2, stained both live and dead cells as long as DNA/RNA is presence in the cells. The dye works by binding to the grove of the DNA in the cells but not the complementary sequence of DNA/RNA of the cells. Detection of specific bacteria works by designing a probe consisting of 18-24 base pair (bp) sequences that targets the 16S rRNA of the ribosomal cells that presence in the desire species of bacteria. A customed design of fluorescently labelled oligonucleotide was used to replaced SYTO 9 for detection of specific species. This research was reported in chapter 3 where a dual stage ITP was used for in-line fluorescence *in situ* hybridisation (FISH) of bacterial cells. A sieving matrix and spacer ions were introduced into the ITP method to separate the excess probe from the hybridised cells-probe complexes based on this different mobilities. The experiments started with optimisation of the counter-pressure with sieving matrix using neutral dye, coumarin 334 as a model study. The optimised conditions used for bacteria detection resulted in a total

analysis time of 30 mins, 13 mins longer than the previous reported method in chapter 2. This is because, an additional step was required to flush the 1.8 % HEC sieving matrix and the LE solution with 2.6 μM probe into the capillary before ITP analysis. Once optimal conditions were achieved, the specificity of the cell detection using the optimised ITP method was examined. The specificty was tested using both *Enterobactericeae* and PsearA probes that targets *E. coli* and *P. aeruginosa* respectively. A signal was obtained using *Enterobactericeae* probe with *P. aeruginosa* cells. A 7 bp match between the *P. aeruginosa* 16S rRNA and the probe sequence was found, explaining the false negative result. However, no signal was observed when PsearA probe was used on *E. coli* cells. This demonstrates that the method can be used for detection of specific species of bacteria depending on the sequence of the probe used. The effect of the probe concentration on signal/peak area was also examined at 26 and 52 μM concentration and found the signal response of hybridisation of the cells-probe increased when the concentration increased. 26 μM was used for further evaluation due to the cost and volume issue but it still provided an adequate signal response. Experiments consist of mix culture of increased *E. coli* concentration with constant concentration of *P. aeruginosa* were conducted using 2.6 μM and 26 μM of PseaerA probe. By normalising the signal response to %, at 26 μM, 90% of the signal was retained in the mixture with less than 50 times excess of *E. coli* concentration as compared to 2.6 μM whereby the signal response drop from 100% to 70% in the similar mixture of *P. aeruginosa: E. coli*. Finally, to benchmark the performance of the in-line staining using ITP, the response signal was compared using off-line stained cells at the same cells concentration. From the peak area (n=3), it appears that 50% of the in-line cells were stained when compared to off-line cells. To verify the results, the off-line and in-line stained cells were filtered onto a black filter for cell counting with a fluorescent microscope. The average total cells count for off-line FISH without ITP was 2.24 x 10^7 stained cells/mL, from 2.50 x 10^7 CFU/mL (based on plate count), indicating the off-line FISH method hybridised ~ 90% of the total cells in solution. The cell count of off-line and in-line cells from ITP band were collected from the outlet vials yield 5.32 x 10^6 cells/mL for in-line ITP and 1.19 x 10^7

cells/mL for off-line further verified the in-line ITP approach stained approximately 50% of the injected intact cells. With 50% of injected cells are stained in-line, the LOD of the in-line ITP approach was 6.0 x 10^4 cells/mL, 3 times higher compared to Lantz *et. al.* [2] due to the reduced staining efficiency. The LOD was confirmed by using the off-line FISH approach at concentration of 3.0 x 10^4 cells/mL yield, similar peak area and height with in-line ITP at 6 x 10^4 cells/mL.

While successful in reducing sample preparation time using this method, the LOD of the in-line approach in comparison to the LOD in chapter 2, is still 2 to 3 order of magnitude higher due to the dye used. This is because, the detection in in-line approach is based on the binding of the probe into the 16s rRNA presented in the ribosomes of the cells. Also, one single custom probe will only bind to one 16s rRNA while in chapter 2, the syto 9 dyes will binds to the groove of the DNA/RNA allowing multiple syto 9 dye molecules to bind in a single DNA/RNA resulting in higher signal intensity for detection. In addition, if the number of ribosomes presented in the cells is small, fewer probes will be able to bind to the 16s rRNA of the cells, thus, decrease the detection limit.

While the dual stage capillary ITP increased the total analysis time to 30 min, the analysis time can be reduced by using microchip and has potential for mass production and portability. This was reported in chapter 4 where the first part of the experiment was conducted using PDMS chip for dual stage ITP of in-line FISH on intact cells. The total analysis time using PDMS chip for in-line FISH cells was reduced to under 4 mins. To ensure that the second ITP band was the cells-probe hybridisation in the chip, an inverted microscope was used during the ITP for visualising and video recording. *E. coli* was stained off-line with SYTO 9 and incubated for 30 min at room temperature before removing excess SYTO 9. Cy5 was used as the fluorophore for the 16S rRNA oligonucleotide. Unlike in the previous chapter, where the spacer ions and terminator ions were in separate vials, the spacer ion was suspended into the terminator solution with bacterial cells. The whole ITP process was visualised using multiband for two different wavelength. From the results, we can verified that the probe did hybridised into the *E. coli* ribosomal RNA and was observed in

the second band. However, one disadvantage of PDMS chip was that, it is not suitable for mass production. Moreover, additional electrodes cast is required to drive the ITP further increases the cost per unit. The second part of chapter 4 was to use 3D printer to print a multimaterial chip with intergrated electrodes to overcome the issues. One limitation of 3D printer is the dimension of the chip in comparison to the photolithography chip. FDM printer with dual extruder was used to print a multimaterial chip with one extruder allocated to print the chip base design and the second extruder used to deposit the conducting PLA into the chip during printing. A straight channel chip with intergrated electrodes was designed using autocad software. The design was converted into STL file before slicing and finally for printing. The printing process of the chip was 2 hours and the dimension of the multimaterial chip was 5 cm x 500 μm x 500 μm. Once printed, the electrodes performance was examined and compared with the in house Pt electrode. The total electrical resistance of the integrated polymer electrodes was calculated to be 0.3 MΩ, corresponding to 40% of the total resistance. While Joule heating was observed when 600 V was applied across the chip. The printed chip is still suitable for ITP because it was able to carry a stable current up to 380 μA. *E. coli* was used as the model strain in this study and the cells were stained off-line with SYTO 9. When voltage was applied across the chip, the cells migrate along the channel forming an ITP band showing the success of multimaterial 3D printed chip for ITP of bacterial cells. In addition, the total cost for a chip was AUD0.50.

In summary, the LOD of *E .coli* using intercalating dye (SYTO 9) was lowered to 78 cells/mL using counter-pressure assisted ITP which is the lowest LOD of bacterial cells using CE that we are aware of. With the addition of a sieving matrix and spacer ions to the ITP method, a dual stage ITP was performed in a single analysis to detect specific species of bacteria by in-line FISH of intact bacterial cells. The total analysis time was 30 min in the capillary system which is four times faster than the standard off-line FISH protocol. Finally, the capillary ITP method was successfully transferred onto two different microchips (PDMS and 3D multimaterial printed chip). This reduced the total analysis time to under 4 mins for the dual stage ITP using PDMS chip while further optimisation is require for the 3D printed

chip for dual stage ITP. The total cost of the 3D printed chip was AUD 0.50 per chip which is cheaper than PDMS chip and can be used for mass production and potential development of a portable system. The work in this thesis therefore, may lead to a new, inexpensive, rapid and specific enumeration method for bacteria.

5.2 Future Directions

This thesis reports the success on using isotachophoresis (ITP) for in-line fluorescence *in situ* hybridisation (FISH) of intact bacterial cells analysed on capillary and microchip. There are some improvements that need to be done. The first priority of any future work in this research is to quantitatively study the in-line FISH of intact bacterial cells with cITP under field amplified condition to further lower the detection limit. Another way to improve the sensitivity of the detection limit is to use a better fluorescent fluorophore such as Alexa or the use of quantum dots fluorophore. Alternatively, peptide nucleic acid (PNA) probe can be used instead of the DNA oligonucleotide for specificity detection. Another area for improvement for the method would be to increase the injected sample volume. This can be achieved by using a larger i.d capillary or longer capillary length.

The second phase would be to try the in-line ITP approach with a real sample such as water or bioligcal samples for example urine or blood. Phung *et al.,* used ITP method to detect bacterial cells in two different water samples by diluting the water samples with the TE solutions and the cells are stained with Syto 9 dye [3]. One of the possible limitations of the ITP method in term of ionic impurities could be, the impact on ITP band stacking if other ionic species with similar mobility with our leader and terminator were presented in the sample. However, this shows no concern if the mobility of ionic impurities do not fall within leader and terminator mobility range.

In the microfluidic area, another essential future focus would be to overcome the hydrodynamic flow in the 3D multimaterial printed chip with integrated electrodes. One possible way is to use Pluronic f-127 as a sieving matrix instead of HEC or PVP. Often, the high viscosity of the sieving matrix is a challenge due to the consequential bubble formation in the channel. This issue was a problem with the use of 1.8% HEC and as a result, 7% PVP was used. Schoch *et al*., uses Pluronic f-127 as a sieving matrix with ITP for selective RNA extraction and separation [4]. The author mentioned that Pluronic F-127 is a thermo-responsive triblock copolymer, which has a low viscosity at low temperature, allowing rapid

microchannel loading. At room temperature, the uncrosslinked Pluronic F-127 will form a liquid crystalline phase that can be used for oligonucleotide separation [4]. This could stop the hydrodynamic flow in the 3D printed chip and also solve the challenge in the loading of the sieving matrix into the channel. Another alternative would be to print a chip with reduced width and depth that can stop the flow. This may successfully lead to the use of the 3D printed multimaterial chip with integrated electrodes for in-line FISH of intact cells. Another potential would be to modify the 3D multimaterial chip design by adding a separate reservoir to fill the bottom channel with sieving matrix.

5.3 References

1. Jasson V, jacxsens L, Luning P, Rajkovic A, Uyttendaele M 92010) Alternative microbial methods: An overview and selection criteria. Food microbiology 27:710-730.

2. Lantz AW, Brehm-Stecher BF, Armstrong DW (2008) Combined capillary electrophoresis and DNA-fluorescence in situ hybridisation for rapid molecular identification of Salmonella Typhimurium in mixed culture. Electrophoresis 29:2477–2484.

3. Phung SC, Nai YH, Powell SM, Macka M, Breadmore MC (2013) Rapid and sensitive microbial analysis by capillary isotachophoresis with continuous electrokinetic injection under field amplified conditions. Electrophoresis 34: 1657–1662.

4. Schoch RB, Ronaghi M, Santiago JG (2009) Rapid and selective extraction, isolation, preconcentration, and quantitation of small RNAs from cell lysate using on-chip isotachophoresis. Lab on a chip 9:2145–2152.